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# SOME QUANTITATIVE STUDIES ON THE MULTIPLICATION OF CONNECTIVE TISSUE CELLS *IN VITRO*.

H. HARRIS.*

*From the Sir William Dunn School of Pathology, University of Oxford.*

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ALTHOUGH it is almost half a century since animal cells and tissues were first cultivated *in vitro*, the sort of quantitative information that is available about bacterial growth still appears to be lacking for tissue culture. The essential reason for this has been the absence, until recently, of an accurate method of measuring cell multiplication in tissue culture. The area of an emigration zone has been used as an index of cell multiplication, but this is open to the objection that this area is determined by the extent of cell migration no less than by the degree of cell multiplication; and even if, as has been maintained, these run *pari passu*, only a very vague idea can be formed of the number of cells originally present in the explant or the proportion of these cells which is viable. Furthermore, cultural conditions in the centre of the emigration zone may be very different from those at the periphery; and it is not possible to assess the influence of substances liberated by the dead cells present. It cannot therefore be assumed that quantitative information obtained by measuring the increase in size of an emigration zone is generally applicable to the multiplication of the cells of which it is composed.

Many of these objections were overcome in the technique devised by Earle and his associates (Sanford, Earle, Evans, Waltz and Shannon, 1951). These workers used suspensions of cells prepared from stock cultures and measured the rate of cell multiplication by direct counts on the number of nuclei present. None the less, Earle's technique has certain disadvantages. The most important of these is that each count involves the destruction of the population being counted, so that serial measurements on the growth of a single population are not possible. Moreover, in order to produce cell suspensions from the stock cultures it is apparently necessary to scrape the cells from the floor of the flasks with a rake (Evans, Earle, Sanford, Shannon and Waltz, 1951). It seems likely that this introduces a number of dead or damaged cells into the preparations, which may account for the irregularity of the growth rate seen during the early stages of some of the experiments (Earle, Schilling, Bryant and Evans, 1954). Finally, it is by no

* In receipt of a personal grant from the British Empire Cancer Campaign.

means certain that stock cultures grown for several years *in vitro* retain their original growth characteristics ; in fact, Earle's experiments on the " production of malignancy *in vitro* " suggest that fundamental changes may take place in such cultures spontaneously (Earle, 1943 ; Earle and Nettleship, 1943).

It seems essential therefore that any method designed to give quantitative information about the multiplication of animal tissue cells *in vitro* should meet the following requirements : The preparations used should be free from dead cells or fragments of dead tissue. The cells should be so disposed that cultural conditions throughout the preparation are uniform. The method must permit serial measurements to be made on the growth of a single population without disturbing that population. These measurements should be made by direct cell counts. Experiments should preferably be carried out as soon as possible after the cells have been isolated from the animal tissues.

In the present study a technique was devised which is thought to fulfil these requirements. The findings reported below have been obtained by its use.

## EXPERIMENTAL.

The method was evolved from a procedure originally devised by Rous and Jones (1916). These authors showed that cells which had emigrated from explants in plasma clot could be released by digesting this clot with trypsin. Most of the cells in the resulting suspension were said to be alive, and could be subcultivated several times. This work appeared to offer a means of obtaining suspensions of viable tissue cells suitable for quantitative studies. But when attempts were made to use the original Rous and Jones procedure for this purpose, it was found that the cell suspensions contained a large proportion of dead cells and more cell débris than whole cells. While the possibility of improving this technique was being investigated, my attention was drawn to the work of Moscona (1952), who had devised a method for obtaining suspensions of cells directly from the tissues by tryptic digestion. It was found, however, that the suspensions obtained by Moscona's method, like those obtained by the method of Rous and Jones, contained large numbers of dead cells. Some experiments reported by Rinaldini (1954), using a slight modification of Moscona's method, showed that more than 50 per cent of the cells obtained in this way were not viable. Preliminary tests carried out with crystalline trypsin made it clear that the dead cells in suspensions prepared by the Rous and Jones or the Moscona technique could be attributed to the action of the trypsin itself and not to any impurities. It seemed reasonable therefore to expect that the percentage of viable cells would be increased if the cells could be brought into suspension with a minimal exposure to trypsin. In the procedure finally adopted it was possible to reduce both the concentration of the enzyme and time over which it acted to such an extent that virtually all the cells in the resulting suspension were viable.

The cells used were undifferentiated connective tissue cells obtained from the hearts of 5-day-old albino rats (Wistar). Each heart was cut up into about 100 small pieces, all of which were explanted on the floor of one Carrel flask of a diameter of 3·5 cm. in the following way : After cutting, the pieces were transferred to a vessel containing 1 ml. of a solution of aged Seitz-filtered rabbit serum diluted 1/5 in Hanks' physiological saline (Hanks, 1948). To this solution crystalline trypsin (Armour), dialysed free of magnesium sulphate, had been added in a concentration just short of that required to neutralise the trypsin-inhibitor present in the serum. The amount of trypsin required to neutralise the trypsin-inhibitor was estimated for each batch of serum by determining the smallest amount of this serum which would completely extinguish the tryptic activity of a known amount of the enzyme. To the vessel in which the pieces of rat heart had been placed 0·2 ml. of chicken plasma was now added. The chicken plasma had been collected in chilled containers and stored frozen until required. The pieces of tissue and the plasma-containing medium were taken up in a wide-mouthed Pasteur pipette and transferred to the Carrel flask ; this was gently shaken so as to distribute the explants more or less evenly. The medium was then withdrawn by means of a fine-pointed Pasteur pipette, leaving the explants anchored to the floor of the flask by no more than a residual film. When this film had clotted, 3 ml. of the diluted rabbit serum

were introduced into the flask. The pH of this medium was adjusted by gassing the flask with a mixture containing 95 per cent $O_2$ and 5 per cent $CO_2$ and the flask sealed. Six to twelve flasks were prepared, depending on the number of cells required for any given experiment.

The preparations were incubated at 37° for 36–40 hr., by which time large numbers of connective tissue cells had emigrated from the explants on the floor of the flasks. These cells were harvested in the following way. The medium was withdrawn and the cultures washed for about an hour with three changes of Hanks' solution in order to remove as much serum protein as possible. A volume of 3 ml. of warmed trypsin solution was then introduced into each flask. The trypsin solution contained 50 μg. of dialysed crystalline trypsin per ml. of Hanks' solution. At this concentration the trypsin released the cells and the explants from the floor of the flask within 10 min. As soon as the cells had been brought into suspension, the contents of the flasks were transferred to chilled vessels, the activity of the trypsin being at once reduced by the rapid fall in temperature. When the fragments of heart muscle had settled out, the supernatant fluid in which the cells remained suspended was taken off and centrifuged at 220 *g* for 3 min. The supernatant was discarded and the cells re-suspended in a small volume of diluted rabbit serum which had been heated for 30 min. at 56°. The density of this cell suspension was determined in a haemocytometer and adjusted as required. The final suspension was introduced into one of two types of culture chamber, in which all subsequent studies were carried out.

The first type was a coverslip chamber 1·5 mm. deep, similar to that devised by Mackaness (1952), except that no centre well was necessary. Chambers of this sort were suitable for short term experiments. After the cell suspension had been introduced into them, they were transferred to an incubator at 37° and not moved again until the cells had become adherent to the bottom coverslip. As the column of fluid in which the sedimentation took place was only 1·5 mm. deep and relatively still, the cells became evenly distributed over the floor of the chamber. It took about 2 hr. for the cells, which were rounded while suspended in the fluid, to resume their characteristic shapes and become firmly adherent to the floor of the chamber. Once this had occurred, it was possible to change the medium without detaching the cells from the coverslip. The medium was changed in all chambers 3 hr. after the cell suspension had been introduced. This removed any extraneous débris which might have been present in the original cell suspension, and left a population of living cells attached to the glass.

The growth of this cell population was measured by marking out several fields on the coverslip with ink in such a way that their position could be exactly identified, and counting the number of cells in these fields at suitable intervals by phase-contrast microscopy. Counts could continue to be made until sheets of contiguous cells were formed. Under the conditions of the present experiments the cells moved continually over the surface of the glass; but this movement was so slow and of such limited extent that it did not introduce any significant error into the counts. After the initial change of medium, the chambers were incubated upside down. This served to separate living from dead cells during the later stages of some of the experiments, when the cells began to die off. Living cells remained attached to the top coverslip and retained their characteristic shape; dying cells became rounded, lost their attachment to the coverslip, fell to the bottom of the chamber and finally disintegrated. All counts thus represented living cells only.

The second type of chamber used is shown in Fig. 1. This chamber was designed to permit adequate oxygenation of the cells over several days and at the same time retain the visual properties of a simple coverslip chamber. Initially, coverslips A and B were sealed with paraffin wax, but the larger coverslip C was not sealed; the lower compartment of the chamber between A and B was thus in effect a simple coverslip chamber similar to the first type used. Into this lower compartment the cell suspension was introduced. The chamber was incubated at 37° until the cells had settled out and become adherent to the lower coverslip A; the medium was then changed, coverslip B removed, and coverslip C sealed. More medium was introduced through the drill holes until the cavity of the chamber was filled except for a large air bubble. The size of this bubble was adjusted so that when the chamber was placed on its side the lower surface of the bubble reached the edge of the peripheral shelf in the chamber but did not encroach on the coverslip to which the cells were adherent. The air bubble was gassed with a mixture of 95 per cent $O_2$ and 5 per cent $CO_2$ and the drill holes were sealed. The chamber was then attached to a vertical turntable in such a position that as the turntable revolved the gas bubble circled round the peripheral shelf. In this way the oxygen tension of the medium was kept in equilibrium with that of the gas phase without

dislodging the cells on the coverslip. The turntable was enclosed in an incubator at 37°, and set at 1 r.p.m. At suitable intervals cell counts were made in the manner described above. If necessary, the gas phase could be renewed or the medium changed.

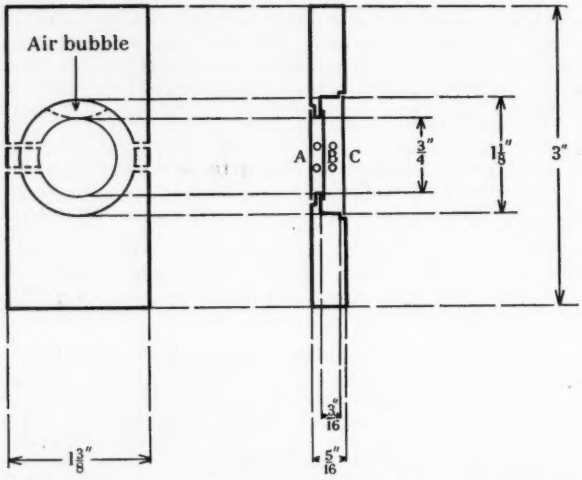


FIG. 1.—Plan of the perspex chamber designed to permit adequate oxygenation of the medium over several days.

## RESULTS AND DISCUSSION.

### *The Effect of Trypsin on Living Animal Cells.*

It has been shown by a number of workers that bacteria, yeasts, unicellular protozoa and certain higher animal forms are not susceptible to the proteolytic action of trypsin while they are alive ; if, however, these organisms are killed they are then readily digested by the enzyme. Findings of this sort have given rise to the general proposition that trypsin cannot act upon living cells ; according to Northrop (1926), the enzyme is unable to penetrate the intact cell membrane. While this proposition may in general be true for free-living organisms, the present investigations seem to show that it is not universally applicable to the somatic cells of animals. Living connective tissue cells, which had emigrated from explants of rat heart, were incubated at 37° in Hanks' solution containing 50 $\mu$g. of crystalline trypsin per ml. The cells were digested within an hour, and the rate of cell destruction could be shown to be proportional within certain limits to the concentration of enzyme used. On the other hand, living leucocytes were found to be relatively insusceptible to the action of trypsin. Polymorphonuclear leucocytes and monocytes obtained from the peritoneal cavity of a rabbit remained alive for many hours in a solution containing 2 mg. of crystalline trypsin per ml.

Trypsin produced certain characteristic changes in connective tissue cells some time before it killed and digested them. These early changes were reversible. Under the cultural conditions adopted in the present experiments the connective tissue cells which had emigrated from the explants of rat heart remained closely adherent to the floor of the flask and could be seen with phase-contrast micro-

scopy to be attached to each other by cytoplasmic bridges. On being exposed to the action of trypsin, these cells gradually withdrew their cytoplasmic processes, became quite round, and eventually lost their attachment to the glass surface. A unicellular suspension was thus obtained. If the cells were now removed from the trypsin solution by centrifugation, and re-suspended in a suitable medium which did not contain the enzyme, they were able to attach themselves once more to a glass surface and resume their usual shapes. If, however, the enzyme was allowed to continue to act, irreversible changes took place which led to the complete disintegration of the cells. The ability of trypsin to detach cells from the surfaces to which they were adherent was not limited to connective tissue cells. Monocytes obtained from the rabbit peritoneal cavity normally adhere very closely to a glass surface at 37° ; but in the presence of trypsin they rapidly lost their attachment to glass. As described above, however, monocytes, unlike connective tissue cells, were not killed by further treatment with trypsin and were able to re-attach themselves to a glass surface and resume amoeboid movement even after many hours' exposure to a high concentration of the enzyme.

After treatment with trypsin, connective tissue cells obtained from rat heart were found to undergo lysis if they were re-suspended in fresh rabbit serum. The rapidity of this lysis was proportional to the concentration of serum used. However, when the rabbit serum was heated at 56° for half an hour, it lost its capacity to destroy the cells. It was for this reason that heat-inactivated serum was used in all experiments. Aged Seitz-filtered heat-inactivated rabbit serum appeared to be free from any observable toxic effects either for cells which had been exposed to trypsin or for cells which had not.

### *General Characteristics of the Multiplication of Connective Tissue Cells in Serum.*

Fig. 2 shows the growth and decline of a population of connective tissue cells in diluted rabbit serum. Before being introduced into the chamber, the medium was saturated at 37° with oxygen, and the pH adjusted to 7·4 with 5 per cent of $CO_2$ in $O_2$. The experiment was carried out in a small coverslip chamber in which no oxygen other than that dissolved in the medium was available to the cells. The medium was not changed at any time during the experiment.

*The lag phase.*

The multiplication of these cells was not preceded by any true lag phase. The cells did not appear able to undergo mitosis while they remained in suspension; since it took about 2 hr. for them to adhere to the floor of the chamber and resume their usual shapes, an apparent lag of this duration was observed. Once the cells had become adherent to the coverslip multiplication began to occur at once. An occasional cell, in which mitosis had apparently begun either before or during the time it was exposed to the action of trypsin, was seen to complete division when it became attached to the coverslip. The absence of any true lag period under the present conditions is not surprising, since the procedure used in these experiments merely involves the dispersion and redisposition of cells which have already begun to multiply. If the behaviour of connective tissue cells in this respect resembles that of bacteria, then a lag phase is hardly to be expected. In the experiments reported by Rinaldini (1954) a " true " lag of about 3 hr. was

said to occur after a growing cell population had been brought into suspension by trypsin. It seems likely that this "true" lag was due either to the time taken by the cells to re-attach themselves to the surface on which they were to be cultivated, as in the present experiments, or to the presence of dead cells in the population as a result of treatment with trypsin.

*The growth phase.*

In serum the growth of the cell population was at no time logarithmic; the growth rate began to fall from the outset and continued to do so until growth

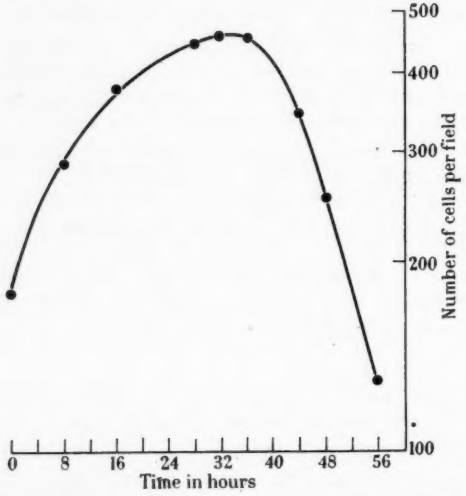


FIG. 2.—The growth and decline of a population of connective tissue cells in diluted serum. The medium was saturated at 37° with oxygen before being introduced into the chamber.

ceased. Under such conditions a mean generation time calculated over the whole period of growth would have little significance; the population doubled its number in about 15 hr. Where the final size of the population was determined by exhaustion of the available oxygen, as is subsequently shown to be the case under the conditions of the present experiment, cessation of growth occurred rather abruptly; with certain other limiting factors, which will be discussed later, cessation of growth was more gradual.

*The stationary phase.*

The period during which there was neither increase nor decrease in the size of the population was not sharply demarcated either from the growth phase which preceded it or from the fall in population which followed it. A well-defined stationary phase such as occurs in the growth of bacterial populations was not observed. When the subsequent fall in population was rapid, the stationary phase was very short; when this fall was more gradual the stationary phase was

more prolonged. The stationary phase was not due to the temporary survival of a cell population in which mitosis had ceased to occur; mitotic activity continued throughout the greater part of the stationary phase, but it was offset by the rate of cell death. Eventually mitotic activity ceased altogether, and the cell population began to fall.

*The phase of decline.*

If the medium was not changed, the population fell in a logarithmic fashion until all the cells were dead. The rate of decline could be altered by varying experimental conditions in certain ways which are discussed in the next section.

### *Effects Produced by Varying the Oxygen Tension.*

The amount of oxygen available to the cells was varied by the following procedures: (1) Before being introduced into the chamber, the medium was saturated at 37° with air, and the pH adjusted to 7·4 with 5 per cent of $CO_2$ in air. (2) The medium was saturated at 37° with $O_2$, and the pH adjusted with 5 per cent of $CO_2$ in $O_2$. (3) The medium was saturated at 4° with $O_2$, and the pH adjusted with 5 per cent of $CO_2$ in $O_2$. At this temperature about twice as much oxygen went into solution as in (2). When the chamber was subsequently incubated at 37° some of the gas came out of solution and formed minute bubbles; these bubbles gradually disappeared during the course of the experiment. (4) The large chamber was used in which the medium was initially saturated with oxygen at room temperature, but in which a reservoir of oxygen was available in the gas bubble. By constantly rotating the chamber equilibrium was maintained between the fluid and gas phases.

The effects of these four procedures on the growth rate of four comparable cell populations is shown in Fig. 3. It will be seen that the initial growth rate was

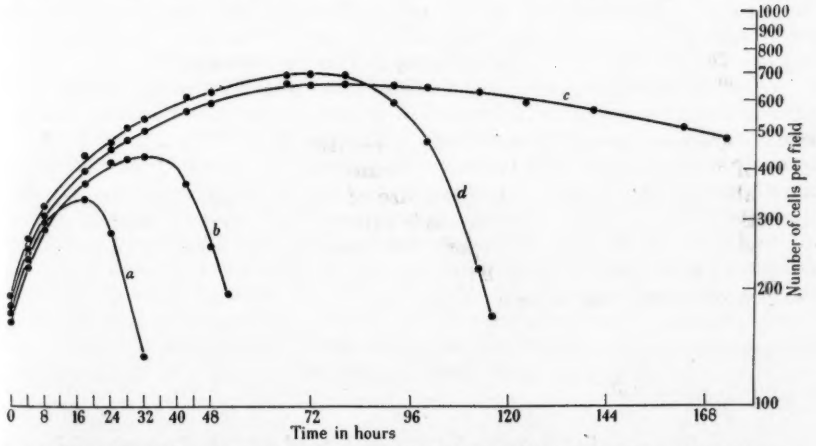


FIG. 3.—The effect of varying the amount of oxygen available to the cells. (*a*) Medium saturated at 37° with air. (*b*) Medium saturated at 37° with oxygen. (*c*) Medium saturated at 4° with oxygen. (*d*) Large chamber.

the same in all four. It is clear, therefore, that, over a range extending from about 20 per cent saturation to 100 per cent saturation, the rate of growth is independent of the oxygen tension. The total amount of growth may, however, be limited by the total amount of oxygen available. Growth ceased sooner in the medium which had been saturated at 37° with air than in the medium which had been saturated at 37° with oxygen, and sooner in the latter than in the medium which had been saturated at 4° with oxygen. In the last, however, cessation of growth was not due to exhaustion of the available oxygen, since the final level attained in this chamber was not less than that attained in the large chamber which contained an oxygen reservoir. When the connective tissue cells were grown in diluted serum alone growth ceased after about 70–80 hr. even though the oxygen supply was adequate. The gradual fall in growth rate and the eventual cessation of growth were found to be uninfluenced by changing the medium, by using a larger volume of medium or by varying the concentration of serum in the medium. The limiting conditions cannot therefore be the exhaustion of some necessary substrates present in the medium or the accumulation in the medium of toxic end-products of metabolism.

When the cells were allowed to multiply in serum until the limit described above was reached, they underwent certain striking morphological and physiological changes. The morphological changes can be appreciated by comparing Fig. 4, which shows the cells 4 hr. after they had become adherent to the floor of the chamber, with Fig. 7, which shows the cells at the same magnification after they had been maintained without change of medium for 172 hr. It will be seen that the cells have become very much larger, or at least they have spread further over the glass. Their outlines have become more irregular and less distinct; well marked cytoplasmic processes have developed between the individual cells; numerous granules have appeared in the cytoplasm; and occasional bi-nucleate cells have been formed. These changes were not due to gradually developing anoxia, since they occurred at the same time in the large chamber which was adequately oxygenated. Although cells which had undergone these changes were no longer able to undergo mitosis, they were able to survive for very long periods if the oxygen tension was low. From Fig. 3 it can be seen that the cell population in the small chamber containing medium saturated at 4° with oxygen declined very slowly after reaching the limit of its growth, despite the fact that the medium was not changed at any time during the experiment and the pH fell to 6·9. The rate of decline in the large chamber containing the oxygen reservoir was much more rapid, even though the population had grown at the same rate and reached the same final density. It would appear, therefore, that cells which have been allowed to reach the limit of their growth in serum and which have undergone the morphological changes described above, are able to survive much better at low oxygen tensions than at high. This conclusion is supported by the finding that a population of cells which had reached the limit of its growth and was declining at a slow rate in a medium with a low oxygen tension could be made to die off much more rapidly if the used medium were withdrawn and freshly oxygenated medium substituted.

*The Effect of Varying the Initial Size of the Cell Population.*

In order to determine whether the initial growth rate was influenced by the "inoculum" size, cell suspensions of varying density were prepared in the same

medium and introduced into a series of small chambers in the usual way. The growth rates of these different populations are shown in Fig. 8. It will be seen that, with the exception of very low inocula, the growth rates were independent of the inoculum size. With dense populations limitation of growth occurred earlier, but the initial growth rate was none the less the same as in smaller populations. This finding is at variance with the observations of Earle, Sanford, Evans, Waltz and Shannon (1951), who found that with mouse connective tissue cells cultivated

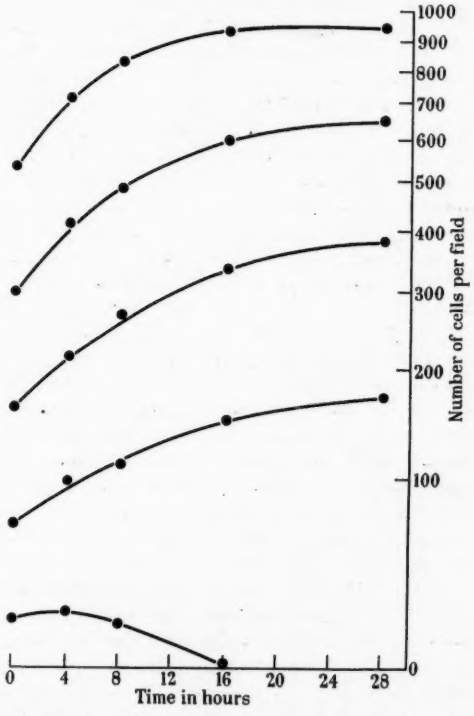


FIG. 8.—The effect of "inoculum size" on the growth rate. The medium was saturated at 4° with oxygen before being introduced into the chambers.

in horse serum and chick embryo extract there was an optimum inoculum size above or below which growth was less rapid. However, in all but one of Earle's experiments the first count of the number of nuclei present was not made until three days after the cells had been introduced into the flasks, by which time limiting factors may already have been operative in the denser cultures; and, as pointed out above, measurements of the early growth rate in Earle's preparations may have been affected by the undetermined number of dead cells present in the original cell suspensions. In the one experiment reported in Earle's paper in which the number of nuclei was counted 24 hr. after the cells had been introduced

into the flasks, it was found that no increase in cell numbers had occurred during this period.

In the small chambers, containing 0·43 ml. of medium, it was not possible to produce growth regularly out of inocula smaller than 4500 to 5000 cells evenly distributed over the floor of the chamber. An evenly distributed inoculum of this size represented approximately 16 to 18 cells per sq. mm. Although a few mitoses were seen to occur in inocula smaller than this, such inocula usually failed to survive. Sanford, Earle and Likely (1948) have suggested that, before a cell population can grow, the medium in which it is disposed must first be " adapted ", and that small inocula fail to grow because they are unable to effect this adaptation. The necessary relationship between the cells and the medium would appear, however, to be more complex than might at first be understood by the term " adaptation of the medium." If it were simply a question of the cells producing certain physical or chemical alterations in the medium, then it might be expected that medium taken from cultures in which the cells were actually multiplying, medium which had therefore presumably been " adapted ", would support the growth of single cells, or at least of much smaller inocula than " unadapted " medium. In the present experiments, however, this was not found to be so. Medium withdrawn from chambers in which the cells had been multiplying for some hours did not support the growth of single cells under the same conditions, or of much smaller inocula than those which were able to grow in ordinary medium. Whether any statistically significant reduction in the minimum viable inoculum size was effected by the use of " adapted " medium was not determined; but certainly no marked difference was observed.

However, a much smaller inoculum relative to the total volume of medium in the chamber could be made to grow if this inoculum were not evenly distributed over the floor of the chamber but confined to a smaller area. It was possible in this way to obtain growth from very small patches of cells provided the density of the cells there was of the order of 20 per sq. mm. or more. The minimum viable inoculum size thus appeared to be more closely related to the density of the cell population than to the ratio of the total population to the volume of medium. In this respect the behaviour of small inocula of connective tissue cells resembled the " nidus " effect seen with certain bacteria. Growth could, however, be obtained from single cells either in " adapted " or ordinary medium if the volume of medium immediately surrounding the cells was reduced to capillary dimensions. When single cells or a few widely scattered cells were cultivated in a capillary space, between a coverslip and a serum agar slab, as described by Pulvertaft (1952),

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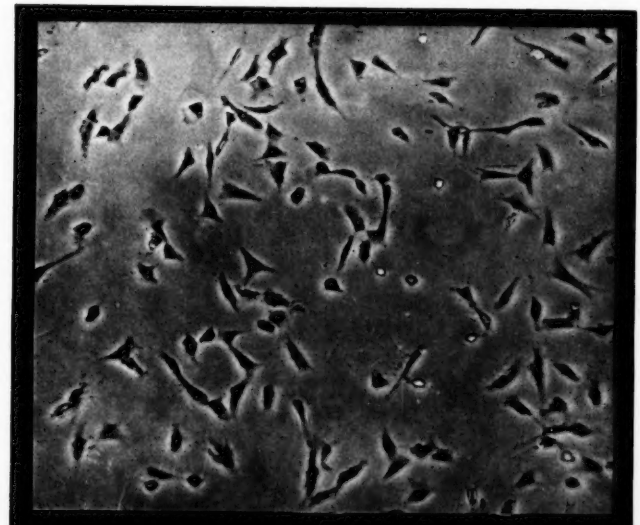
EXPLANATION OF PLATES.

FIG. 4.—The connective tissue cells 4 hr. after they had become adherent to the floor of the chamber. This preparation contains approximately 65 cells per sq. mm. × 64.

FIG. 5.—The cells 36 hr. after they had become adherent to the floor of the chamber. This preparation contains approximately 170 cells per sq. mm. × 64.

FIG. 6.—The cells 120 hr. after they had become adherent to the floor of the chamber. The population has begun to decline. The cells have spread out over the glass and are apparently larger; their outlines are less distinct. This preparation contains approximately 150 cells per sq. mm. × 64.

FIG. 7.—Cells maintained at a low oxygen tension without change of medium for 172 hr. The changes shown in Fig. 6 are more pronounced and the cytoplasm of the cells contains numerous granules. × 64.



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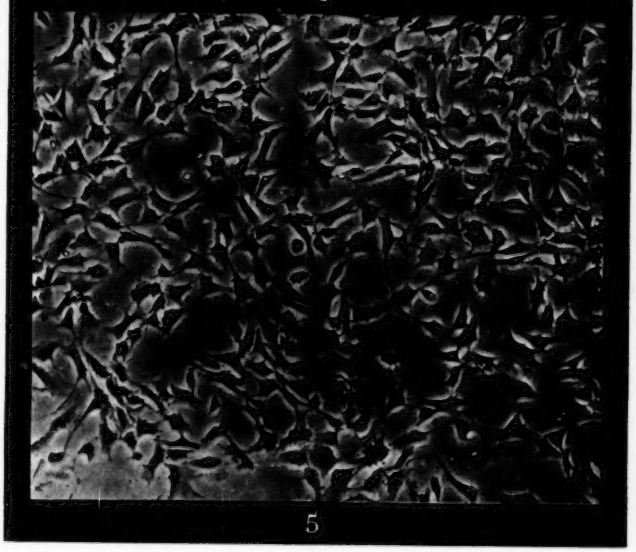

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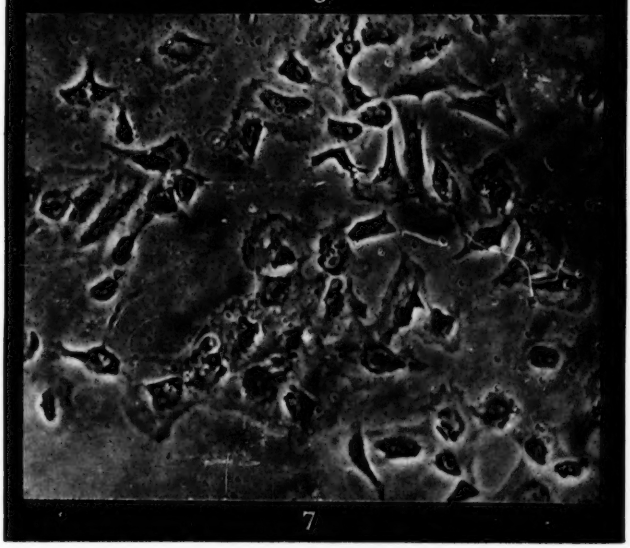

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it was found that each of the isolated cells usually gave rise to a small colony. This is in agreement with the observations of Sanford *et al.* (1948) who obtained growth from single cells isolated in fine capillary glass tubes.

*The Effect of Varying the Concentration of Serum in the Medium.*

Comparable cell populations were grown in the small chambers in concentrations of serum ranging from 5 to 80 per cent in Hanks' solution. The initial growth

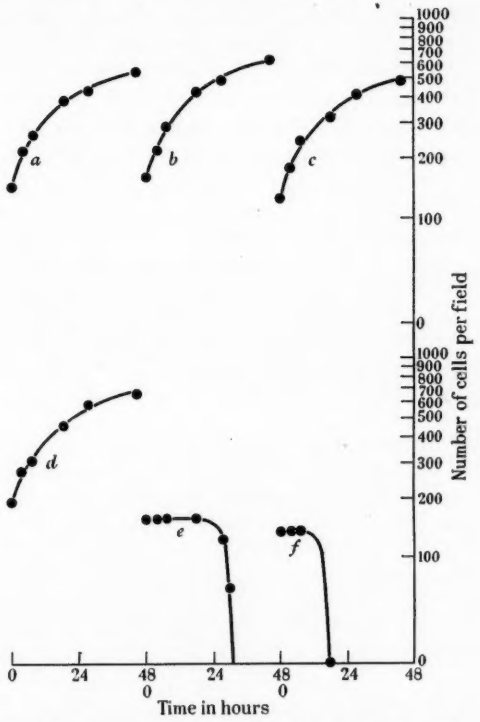


FIG. 9.—The effect of serum concentration on the growth rate. (*a*) 80 per cent of serum in Hanks' solution. (*b*) 60 per cent of serum. (*c*) 40 per cent of serum. (*d*) 20 per cent of serum. (*e*) 10 per cent of serum. (*f*) 5 per cent of serum. All media were saturated at 4° with oxygen before being introduced into the chambers.

rates of these populations are shown in Fig. 9. It will be seen that above a certain minimal concentration of serum which, with the aged Seitz-filtered serum used in the present experiments, usually lay between 10 and 15 per cent, the growth rate was independent of the serum concentration. This finding is essentially in accord with the observations of Monod (1942) on the growth of bacterial populations under similar conditions. However, the behaviour of connective tissue cells in concentrations of serum below the minimum necessary for multiplication appeared to be different from that of bacteria in very low substrate concentrations.

According to Monod, bacterial cultures do not have any " maintenance " requirements ; so far as is measurable, the consumption of even the smallest amount of substrate results in a corresponding amount of growth. On the other hand cultures of connective tissue cells which did not grow in 5 or 10 per cent of serum nevertheless maintained their numbers in these concentrations for many hours. Those in 10 per cent of serum survived longer than those in 5 per cent ; and when death finally occurred, it took the form of a quite rapid disintegration of the whole population. In Hanks' solution without serum the cells died in about 1 hr. at 37°. These findings might be interpreted as showing that connective tissue cells, unlike bacteria, do have a " maintenance " requirement, which may be met, at least for some time, by concentrations of substrate below those which support cell multiplication.

*The Effect of Changing the Medium.*

If, during the growth phase, the medium was withdrawn and replaced by fresh medium warmed to 37°, growth continued without any measurable interruption. Cells in which mitosis had already begun were seen to complete division, and others to enter mitosis, immediately after the change. No lag or fall in the mitotic rate occurred ; the growth curve in the period immediately following the change of medium was no different from that of control cultures in which the medium was not changed. This was so even when the cells were washed several times by repeated changes of medium. This finding would appear to provide strong evidence against the view that when a growing culture of connective tissue cells is transferred to fresh medium, the medium has to be " adapted " before growth can continue.

When the cells were grown under conditions in which exhaustion of the available oxygen was the limiting factor, changing the medium before this limit was reached allowed more growth to occur than would otherwise have been the case. Since changing the medium introduced additional oxygen into the system, this result was to be expected. However, once the cell population had begun to decline as a result of oxygen lack, this decline could not be arrested or retarded by changing the medium. Apparently the injury produced by acute anoxia is irreversible. As mentioned above, the limitation of growth which occurred in serum despite the presence of adequate oxygen could not be prevented by previous changes of medium.

## SUMMARY AND CONCLUSIONS.

The techniques developed in this investigation have made it possible to measure the multiplication of mammalian tissue cells *in vitro* with a degree of accuracy comparable to that obtainable in studies on bacterial growth. The present paper reports certain preliminary measurements made with these techniques on the multiplication of rat connective tissue cells in serum.

When grown in serum, these cells did not exhibit any true lag phase, the growth phase was at no time logarithmic, and the rate of growth began to fall from the outset and continued to do so until growth ceased. A well defined stationary phase such as occurs in the growth of bacterial populations was not observed. There was a variable period at the end of the growth phase during which the rate of cell multiplication was approximately balanced by the rate of cell death.

If the medium was not changed, the population fell in a logarithmic fashion

until all the cells were dead. The rate of decline could be altered by varying the experimental conditions. Over a wide range, the growth rate was independent of the oxygen tension ; but under certain conditions the total amount of growth which occurred was determined by the total amount of oxygen present. In serum growth ceased after about 70–80 hr. even in the presence of an adequate oxygen supply. The gradual fall in growth rate and eventual cessation of growth were uninfluenced by changing the medium, by varying the volume of medium or by varying the concentration of serum in the medium. Cells which were cultivated in serum until multiplication had ceased underwent gross morphological and physiological changes. At low oxygen tensions such cells were able to survive for many days ; at higher oxygen tensions they rapidly disintegrated.

The growth rate was independent of the inoculum size except for very small inocula from which it was difficult to obtain any growth at all. The minimum viable inoculum size appeared to bear some relationship to the density of the cell population ; it was not substantially reduced by using medium in which some cell multiplication had previously taken place. Growth could be obtained from a single cell only if the volume of medium surrounding the cell was reduced to capillary dimensions.

The growth rate was independent of the concentration of serum in the medium above a certain minimum. If, during the growth phase, the medium was withdrawn and replaced by fresh medium, growth continued without any measurable interruption. The growth curve in the period immediately following the change of medium was no different from that of control cultures in which the medium was not changed.

It is a pleasure to acknowledge my indebtedness to Sir Howard Florey, F.R.S., in whose department this work was carried out. Thanks are also due to Mr. G. J. Wren for technical assistance, and to Mr. B. H. Glass for some help with the photography.

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# THE INFLUENCE OF THE ROUTE OF INJECTION ON LETHAL INFECTIONS IN MICE.

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It is widely held that localisation in the tissues of invading bacteria is of advantage to the host, and, with bacteria causing fatal generalised infections, that localisation is essential for survival. This concept is explicit in Menkin's "inflammatory fixation," summarised in the remark: "A first essential for the successful resistance to infection is that it should be localised—the wider the spread and the larger the area of tissue involved, the greater the risk to life" (Hadfield and Garrod, 1947). This statement implies that dissemination of the bacteria is not of advantage to the host.

The last and perhaps the most important phase of dissemination is invasion of the blood stream, an effect which can be imitated experimentally by intravenous injection of bacteria and compared with the effect of introducing them into the superficial tissues. Comparisons made by previous workers have shown that the tissues reached by bacteria introduced into the blood can dispose of some bacteria with greater facility than, *e.g.*, the subcutaneous tissues. These results suggest that localisation is not necessarily the best reaction for survival of the infected animal.

Thus Sobernheim and Murata (1924) observed that the minimum fatal dose of a strain of *B. anthracis* for guinea-pigs was ten times greater when given intravenously than when injected by the subcutaneous or intraperitoneal route. Lange and Gutdeutsch (1928) tested a pasteurella, a streptococcus and a pneumococcus by the same routes in mice; they recorded the highest death rates after intraperitoneal and the lowest after intravenous injection, subcutaneously injected bacteria being intermediately lethal. Ørskov (1940) records that whereas as few as 5 Type I pneumococci were fatal in mice by the intraperitoneal route, 50,000 were required to kill 8/10 mice by the intravenous route.

The experiments described in the present paper were aimed at extending these investigations by using a wide range of mouse-pathogenic bacteria causing generalised infections. The same three routes of injection were used, the intravenous injections (IV) representing a rapidly disseminated infection, the subcutaneous (SC) and the intraperitoneal (IP) injections offering opportunities of two different kinds for the mobilisation of localising defences.

## MATERIALS AND METHODS.

*Animals.*—Healthy mice were used of the Institute "Parkes" albino strain. Pregnant animals or those with scratched skin were rejected. The weight range of the mice in all the experiments was 25–35 g., and individual adjustment of dose was avoided by restricting the range to 5 g. in any one experiment.

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*Bacteria.*—Fifteen species representing eleven genera were tested for mouse pathogenicity. One strain each of *Pasteurella pseudotuberculosis*, *Micrococcus tetragenus* and *Actinomyces muris* and several strains of *Pseudomonas hydrophila* and *Staphylococcus pyogenes*, proved of insufficient virulence. The ten species used (representing eight genera) were *Streptococcus pneumoniae* Type I (NIMR strain), *Diplococcus mucosus** (NCTC 8101), *Erysipelothrix rhusiopathiae* (NIMR (duck) strain), *Staph. pyogenes* (Malmo 30/30), *Klebsiella pneumoniae* Type A (NCTC 5054), *Bacillus anthracis* (Spore suspension, Microbiological Research Department, Porton), *Salmonella typhi-murium* (NIMR strain), *Pasteurella septica* (NCTC 7460), *Streptococcus pyogenes*, Lancefield group C (CN 771 in the Wellcome collection) and an enterococcus (*Streptococcus faecalis*) " BP ". This last organism was isolated by Gledhill and Rees (1952) from the livers of mice suffering from an enterococcal epizootic. According to these workers, it differs from typical *Str. faecalis* in its inability to ferment mannitol ; it also fails to ferment sorbitol, to grow at 45°, to hydrolyse aesculin or to tolerate potassium tellurite.

*Cultures.*—With the exception of the spore suspension of *B. anthracis* all the organisms were mouse-passaged to enhance virulence. Heart blood from moribund animals was cultivated in horse blood broth and incubated for 18 hr. at 37°. The young cultures were then washed and suspended in 2 per cent peptone water diluted 1/10 in saline. Tenfold dilutions were made and counted by the method of Miles and Misra (1938) immediately before and after inoculation of the batch of mice under test.

*Injections.*—Except where otherwise stated, the volume of all inocula was 0·25 ml. The SC injections were made in the lower left lumbar area, IV injections were made into a tail vein, discarding all animals in which any subcutaneous leaks were suspected. Each bacterial dilution was injected into 30 mice, *i.e.*, 10 mice by each route, except with the enterococcus where groups of 20 mice were used. The mice were examined twice daily for at least ten days to record sickness or death. With the exception of deaths caused by the enterococcus "BP", specific infective death was recorded only when both heart blood cultures and Gram-stained smears from the cut surface of the heart were positive. The enterococcal infection rarely gave rise to positive heart blood culture and the bacteria were infrequently detected in the blood smear ; specific death due to this organism was however established by culture of material from the liver lesions.

## RESULTS.

The death rates with nine of the mouse pathogens are shown in the Figure and those with *Kl. pneumoniae* in Table I.

TABLE I.—*Changes in Virulence of an Attenuated Strain of* Kl. pneumoniae *after Mouse Passage.*

| Number of mouse passages. | Dose of living bacilli. | Number of mice dying per group of 10. IP. | SC. | IV. |
|---|---|---|---|---|
| 0 | $10^8$ | 2 | 0 | 0 |
| | $10^9$ | 8 | 2 | 4 |
| 1 | $10^8$ | 4 | 2 | 10 |
| | $10^9$ | 8 | 3 | 10 |
| 2 | $10^8$ | 9 | 0 | 9 |
| | $10^9$ | 10 | 1 | 10 |

Six of the ten organisms tested were lethal to varying degrees in doses of $10^5$ organisms or less. The pneumococcus and pasteurella proved the most virulent,

* The organism *Diplococcus mucosus* is so named in this paper because it is thus listed by the National Collection of Type Cultures although its systematic position is still in doubt. Known also as *Diplococcus mucosus* von Lingelsheim it is placed by Bergey's Manual of Determinative Bacteriology in the family *Neisseriaceae*, Genus I, Appendix I. It is an aerobic Gram-negative coccus occurring in pairs and tetrads : most strains are said to be pathogenic to mice (Wilson and Miles, 1946).

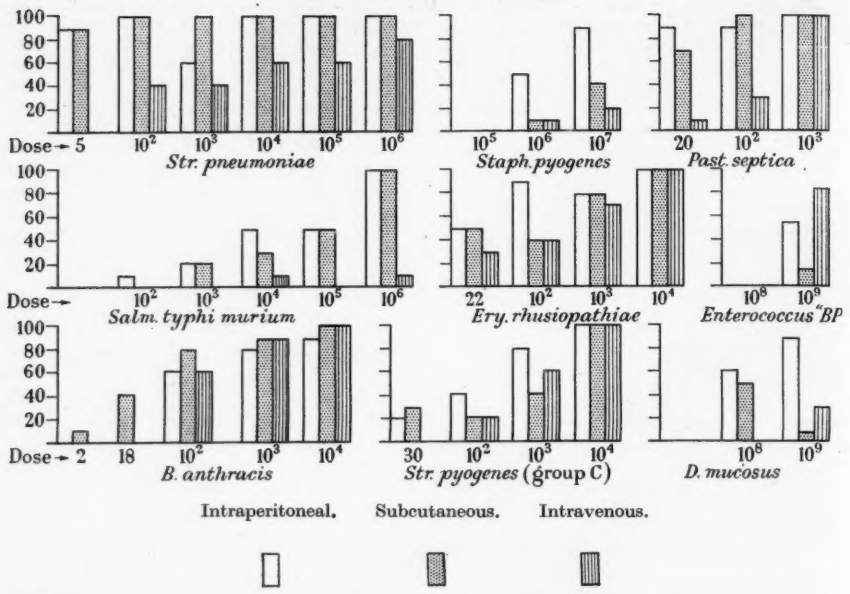


FIGURE.—Final percentage death rate resulting from intraperitoneal, subcutaneous and intravenous injection of pathogenic bacteria in mice. The dose increments were exactly ten-fold, but doses exceeding 50 organisms are recorded to the nearest power of 10.

the Group C streptococcus, *Ery. rhusiopathiae* and *B. anthracis* were somewhat less virulent, and to about the same degree, and *Salm. typhi-murium* was materially the least virulent especially by the IV route. The minimum lethal dose by any route for the *Staph. pyogenes* and *D. mucosus* was approximately $10^6$ and $10^7$ organisms respectively, for the *Kl. pneumoniae* $10^8$ and for the enterococcus " BP " $10^9$ organisms.

With the exception of the streptococcus " BP " and *Kl. pneumoniae* all the strains were less lethal by the IV than by either the SC or IP routes. The difference was only slight with the group C streptococcus and with *Ery. rhusiopathiae*, the IV death rate being consistently lower than the IP death rates at all response levels less than 100 per cent. With both organisms the mean time to death was longer in IV-injected mice than in the other groups (Table II).

The lower lethality of organisms when injected intravenously was striking with the Type I pneumococcus, *Past. septica* and particularly so with *Salm. typhi-murium*. There is little to choose between the lethality of organisms by the IP and SC routes but on the whole the IP is the more lethal especially with those organisms requiring large doses to kill. The order of lethality is largely confirmed by the mean survival time of those that died. These are recorded in Table II for the smallest dose of bacteria killing 80 per cent or more of the mice by at least one route.

TABLE II.—*Mean Time to Death of Mice Inoculated by Intraperitoneal (IP), Subcutaneous (SC) and Intravenous (IV) Routes.*

| Species. | Approximate living dose organism. | Route of injection. IP. Number of mice dying. | IP. Days to death. | SC. Number of mice dying. | SC. Days to death. | IV. Number of mice dying. | IV. Days to death. |
|---|---|---|---|---|---|---|---|
| *Str. pneumoniae* | 50 | 10 | **2·0** | 10 | 3·7 | 4 | 4·0 |
| *Str. pyogenes* (Group C) | $10^3$ | 8 | 4·5 | 4 | **4·0** | 6 | 5·5 |
| Enterococcus " BP ". | $10^9$ | 11 | 4·4 | 3 | **4·3** | 17 | 7·3 |
| *Ery. rhusiopathiae* | $10^2$ | 9 | 6·0 | 4 | **4·5** | 4 | 5·7 |
| *Past. septica* | 20 | 9 | **1·0** | 7 | 2·3 | 3 | 2·0 |
| *Salm. typhi-murium* | $10^6$ | 10 | 3·6 | 10 | **2·1** | 1 | 5·0 |
| *B. anthracis* (spores) | $10^2$ | 6 | 4·0 | 8 | **3·6** | 6 | 4·5 |

The shortest death time for each organism is shown in heavy type. Three species have been omitted, the mean time to death being < two days.

The pneumococcus is interesting in that the dose response IV is very shallow as compared with the steep rise in mortality by the SC and IP routes by which less than 10 cocci were fatal. Neither the enterococcus nor *Kl. pneumoniae* was less lethal by the IV route. In fact for both the IV injections were the most lethal and the SC the least so. The survival time of the mice dying of the enterococcal infection was nevertheless greatest after IV injection. The survival time following the injections of *Kl. pneumoniae* was <two days in all three groups. This fact, taken with the very large dose of organisms required to kill, suggested that death might be predominantly toxaemic rather than infective. The point was investigated by comparing, in groups of 30 mice, the lethality by each of the three injection routes of $10^9$ living *Kl. pneumoniae* and similar suspensions killed (1) by heating for 30 min. at 55° and (2) by holding for 18 hr. at 4° with formalin. The per cent death rates with the living cultures were IP 90, SC 10 and IV 100. Of the mice given heat-killed cultures only one died; all given formol-killed bacteria survived.

Although a direct toxaemic effect on the reticulo-endothelial cells could be excluded it seemed possible that the massive doses of *Kl. pneumoniae* required to kill might overload the cells to the point where their bactericidal power was impaired and so promote lethal infection.

To test such a possible " blockading " effect a mixed suspension of $2{\cdot}5 \times 10^8$ formol-killed *Kl. pneumoniae* and 63 living Type I pneumococci was injected IV into 10 mice. A second group received the same dose of killed *Kl. pneumoniae* with 6 pneumococci. These suspensions of bacteria were injected in 0·5 ml. volumes as soon as possible after mixing. At the same time the two doses of pneumococci were tested alone by the IV, IP, and SC routes. The results (Table III) were clear cut—the killed *Kl. pneumoniae* did not potentiate the pneumococcal infection.

The IP and SC results with pneumococci alone confirm the greater lethality of the bacteria when given by these routes. A moderate impairment of the clearing mechanism by living *Kl. pneumoniae* was however demonstrable. The strain was attenuated by daily successive subculture in broth, and its virulence then raised by two mouse passages. The results (Table I) indicate a substantial

TABLE III.—*The Effect of a Simultaneously Administered Dose* ($2{\cdot}5 \times 10^8$) *of Killed* Kl. pneumoniae *on a Type I Pneumococcal Infection.*

| Dose of living pneumococcus. | Number of mice dying per group of 10 after inoculation with: Pneumococcus. IP. | Pneumococcus. SC. | Pneumococcus. IV. | Pneumococcus and Klebsiella. IV. |
|---|---|---|---|---|
| 63 | 10 | 10 | 1 | 0 |
| 6 | 10 | 8 | 0 | 1 |

rise of IV virulence after one passage and of IP virulence after two passages. These two strains, the attenuated (A) and the more virulent double passage strain (P) were used in mixed IV infections with the Type I pneumococcus an an attempt made to compare the rate at which the bacterium and the pneumococcus were removed from the blood. The following mixtures were tested:

(1) *Kl. pneumoniae* phase "A" (0·25 ml. of $8{\cdot}25 \times 10^8$/ml.) *plus* an equal volume of *Str. pneumoniae* culture ($4{\cdot}4 \times 10^9$/ml.) and

(2) *Kl. pneumoniae* phase "P" (0·25 ml. of $5 \times 10^8$/ml.) *plus* an equal volume of the same *Str. pneumoniae* culture. The relative degrees of bacteriaemia were measured 5 and 120 min. after intravenous injection. Two groups each of 8 mice received respectively the "A" and "P" mixtures. A peripheral (tail) bleeding of such lethally infected and therefore rather shocked mice being difficult, half the mice in each group were anaesthetised at the appropriate times and were bled out from the axillary vein. The blood from each mouse was diluted in peptone water diluted 1/10 in saline and containing 0·05 per cent "Liquoid" (sodium polyanetholsulphonate) to counteract the bactericidal power of the blood. Equal volumes of these samples were pooled and four pour plates in layered blood agar made from tenfold dilutions of each pool. In addition samples of liver and spleen were removed for histological examination from all the mice killed after 120 min.

As Table IV shows, with both the attenuated and passage strain of *Kl. pneumoniae*, the number of viable organisms after 120 min. was between 2 and 6 times that after 5 min., but the attenuated strain was less abundant than the passage strain, indicating a less effective clearing of the latter by the reticulo-endothelial system.

TABLE IV.—*The Effect of Attenuated* (*A*), *and Passaged* (*P*) Kl. pneumoniae *on the Degree of Bacteriaemia after Intravenous Injection of a Pneumococcus.*

| Strain of *Kl. pneumoniae.* | Dilution of blood. | Number of Pneumococci after (min.). 5 | Pneumococci after (min.). 120 | *Kl. pneumoniae* after (min.). 5 | *Kl. pneumoniae* after (min.). 120 | Proportionate change in number of bacteria in peripheral blood after 115 min. Pneumococci. | *Kl. pneumoniae.* |
|---|---|---|---|---|---|---|---|
| A | $10^{-5}$ | 514 | 15 | 100 | 353 | 0·03 | 3·53 |
| | $10^{-6}$ | 61 | 5 | 16 | 38 | 0·08 | 2·38 |
| P | $10^{-5}$ | 464 | 53 | 140 | 770 | 0·11 | 5·50 |
| | $10^{-6}$ | 57 | 12 | 22 | 91 | 0·21 | 4·14 |

In both series of experiments the pneumococcus appears virtually to be cleared from the blood, the counts dropping by 5- to 30-fold; but the clearing is less efficient in the presence of the more virulent *Kl. pneumoniae*. It was not possible to make an accurate count of the relative numbers of bacilli and cocci taken up by the macrophages of the liver and spleen sinusoids as seen in Giemsa-stained sections of these organs removed from the animals killed 120 min. after injection, but substantially greater numbers of bacilli were lying free in the sinusoids of the liver in mice infected by the passaged strain than in those infected by the attenuated strain. These results are consistent with the view that the very large doses of living bacilli of the strains of *Kl. pneumoniae* used were peculiarly effective when given intravenously, because they directly impaired certain antibacterial functions of the reticulo-endothelial system; the same explanation may well apply to the results with the " B.P " enterococcus. Whether or not this explanation be correct, the fact remains that of the species of bacteria tested only two were more lethal by the intravenous than by the other two routes, and both these organisms were so avirulent that they were wholly ineffective by any route in doses of less than $10^8$ living organisms.

## DISCUSSION.

The results largely confirm those of Sobernheim and Murata (1924), Lange and Gutdeutsch (1928) and Ørskov (1940), particularly with regard to the lesser lethality of the intravenously injected, as compared with subcutaneously or intraperitoneally injected organisms. The difference with the Group C streptococcus was not as striking as that recorded for a streptococcus by Lange and Gutdeutsch.

These workers observed that subcutaneous injections of *Ery. rhusiopathiae* were more lethal than those given IV or IP.

Tenbroeck (1920) reported that organisms of the genus *Erysipelothrix* have the property of multiplying in phagocytic cells and of causing a leucopenia. This phenomenon is possibly the explanation for the observation that, with this organism, relatively lower death rates did not occur with IV-injected mice. It will be seen from Table I that death rates by the IV route were higher, using mouse-passaged *Kl. pneumoniae*, than were the SC or IP death rates. However, when the organism was attenuated by cultivation in broth much of the virulence was lost and IV injections were again less lethal than IP.

Five of the strains tested, *Staph. pyogenes*, *D. mucosus*, *Kl. pneumoniae*, the Group C streptococcus and the enterococcus " BP " were more lethal by the IP than by the SC route. To these can probably be added the pneumococcus although virtually all the mice in these groups were killed, even with doses of $< 10$ organisms. At all dose levels with this organism the mean time to death in the IP groups was substantially shorter. The higher IP death rates suggest that the peritoneal defences may be less capable of inactivating some bacteria (at least in the doses used) than are the defences in the subcutaneous tissues. Bacteria injected SC have been shown to be distributed over a very small area (Hewitt, 1954). Peristalsis is likely to disperse IP-injected organisms through the peritoneal cavity within a short time after injection, thus creating multiple foci of infection. Nevertheless a proportion of the organisms is early removed to the blood stream *viâ* the diaphragmatic lymphatics (Durham, 1897). This may well account for the relatively low IP death rates obtained with *B. anthracis* for it is

possible that spores fail to set up inflammatory reactions in the peritoneal cavity and that those not having yet germinated are largely removed to the blood stream and thence to relatively resistant tissues.

The three remaining organisms, *Past. septica*, *Ery. rhusiopathiae* and *Salm. typhi-murium* were about equally lethal by both the IP and SC routes. Lange and Gutdeutsch (1928) using *Ery. rhusiopathiae* reported higher death rates by the SC than by either IP or IV routes.

The variation of lethality with route of administration is apparently a phenomenon of nonspecific immunity. Specifically immune animals were not investigated but it is possible that the mice were partially immune to both the *Salm. typhi-murium* and the enterococcus because both organisms had in the past appeared as sporadic pathogens in the Institute stock mice and because both proved to be relatively avirulent. The lower lethality by the IV route may therefore hold in specifically immune animals providing massive doses are not required to kill.

With the exception of the pneumococcus and the pasteurella, none of the strains tested was highly mouse-virulent in the sense that a few viable organisms are sufficient to establish a certainly lethal infection. For this reason they may be accepted as resembling the organisms producing natural infections more nearly than highly virulent strains, because the prevalence of infecting strains of this kind in nature would rapidly lead to elimination of a susceptible species of host; nevertheless, like the more " natural " moderately virulent strains, the more highly virulent were also least lethal by the intravenous route.

The implications of these results are various. They emphasise the dependence of measures of virulence, when made in terms of death rates, on the route of injection. Not only does the $LD_{50}$ vary with route but the slope of the dose response may do so also. They also emphasise the comparatively high defensive value of the phagocytes of the reticulo-endothelial cells bordering the blood stream and hence raise the question of the value of localisation as a defence mechanism. The significance of these experiments in estimating the survival value of the various modes of defence in natural infections is, however, less clear. If the subcutaneously and intravenously injected mouse can be regarded as models respectively for a natural infection with primary lodgement in a subepithelial tissue and a stage of infection when the invasion from the primary lodgement has reached the blood stream, then clearly the more quickly certain bacteria reach the blood stream, in the presumably small numbers that initially invade the body, the greater the possibility of the host's survival. But direct experimental intravenous infection differs in several respects from the way in which bacteria reach the blood stream from a natural primary lodgement. Bacteria from such sites are removed either free or within macrophages to lymphatics and thence to lymph nodes where many are destroyed. If however the number of organisms is large or there is a considerable increase in the flow of lymph such as accompanies the early stages of inflammation (Lassar, 1877) material numbers of bacteria may filter through into the blood stream. What then may be said in favour of and against the localisation of bacteria at the site of primary lodgement, bearing in mind the statement quoted at the beginning of this paper ? Localisation is likely to be an adequate defence when either the number of bacteria is fairly small and they are not of high virulence for that host, or else, the infecting dose being somewhat larger and the host possessing some degree of immunity, an early and adequate humoral defence

is possible. In some instances phagocytes, and perhaps antibodies also, may fail to achieve sufficient access to bacteria localised by a barrier of fibrin behind which the organisms multiply until they cease to be contained and overwhelm the defences of the host.

There will be some cases where dispersal locally in the tissues favours the host. "The initial establishment of a lesion depends on the concentration of bacteria per unit area of tissue rather than on their absolute numbers" (Hadfield and Garrod, 1947). As shown by Duran-Reynals (1935), where dispersal reduces the concentration of bacteria below the minimum infecting dose per unit area this event may itself enable the body to overcome the infection.

Whether or not early localisation of the organisms is essential is difficult to assess in any particular case. In naturally occurring infections the original infecting dose and the course of events which would follow in the absence of localisation are unknown. The evidence from these experiments is too slender for generalisation to other kinds of pathogen or to other species of host. Nevertheless, the superior resistance of the tissues reached by intravenous injection to all the strains tested except those which infect only in massive doses, warrants a reconsideration, in some infections at least, of the doctrine that localisation of the invading organism at its primary lodgement is necessarily the most effective defence reaction.

SUMMARY.

Ten species of mouse-pathogenic bacteria representing eight genera were injected into mice by the intraperitoneal, subcutaneous and intravenous routes and the death rates recorded.

Eight of the species were less lethal when injected intravenously than by the other two routes especially in the lower doses probably equivalent to natural infections.

The remaining two species, an enterococcus and a Type A *Kl. pneumoniae*, killed only in very large doses and were most lethal when injected intravenously. Experiments with *Kl. pneumoniae* suggested this might be due to impairment of the antibacterial functions of the reticulo-endothelial system by the large number of organisms injected.

The order of lethality of the injections by the three routes was largely confirmed by the mean times to death of the mice.

The lower intravenous death rates observed for the majority of the species tested suggest that in certain infections early removal of bacteria from the subcutaneous tissues to the lymphatics and blood stream may be of advantage to the infected host. This warrants a reconsideration of the doctrine that localisation of organisms at the site of primary lodgement is necessarily the most effective defence against infection.

I am greatly indebted to Professor A. A. Miles for his help and advice in this work which was carried out in his department.

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# ISO-IMMUNISATION OF RABBITS BY MEANS OF FREUND-McDERMOTT ADJUVANTS.

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THE purpose of the experimental work which follows was to develop, in rabbits, anti-erythrocytic iso-antibodies of high titre. When it became apparent that multiple intravenous injections of red cells did not readily achieve this, Freund-McDermott adjuvants were employed, and the results are considered to be of sufficient general immunological interest to warrant publication.

Since Lewis and Loomis (1924, 1925) reported that tuberculous animals responded to injections of an antigen by greater production of circulating antibody than did normal animals, numerous attempts have been made to enhance antibody response by injection of "adjuvants" containing tubercle bacilli. The first reported use of an oily suspension of tubercle bacilli for this purpose is that of Landsteiner and Chase (1940), who succeeded in sensitising guinea-pigs to a simple chemical compound, picryl chloride, by injecting it into the peritoneal cavity of guinea-pigs which had previously received an intraperitoneal injection of killed tubercle bacilli suspended in liquid paraffin. In 1942 Freund and McDermott described a method of immunisation in which the antigen (horse serum) was emulsified in an oily suspension of heat-killed dried tubercle bacilli, using Aquaphor—an emulsifying agent—to obtain a stable emulsion. A single injection of this emulsion, containing 0·125 ml. of horse serum, resulted in the development of large amounts of precipitin. Further reports describe the successful use of the adjuvant emulsion technique to develop antibodies against a variety of antigens, including diphtheria toxoid, killed typhoid bacilli (Freund and Bonanto, 1944), killed influenza virus (Friedewald, 1944), ragweed pollen extract (Kulka and Hirsch, 1945), and killed malaria parasites (Freund, Thomson, Somner, Walter and Schenkein, 1945). The animals used in these experiments include guinea-pigs, rabbits and monkeys, and the results suggest that the antigen-adjuvant emulsions were stronger immunising agents than the antigens alone, and provided a method of stimulating, by a short course of injections, the development of antibodies of high titre. The above reports, together with those of Freund, Somner and Walter (1945) and Lewis, Paterson and Smithwick (1950), suggest that modifications in the composition of the adjuvants are without advantage. Saprophytic acid-fast bacilli are as effective as tubercle bacilli, whereas non-acid-fast bacilli are much less effective. Several proprietary brands of lanolin-like emulsifying agents have been used, apparently without influencing the immunity response. The use of peanut oil or sesame oil in place of liquid paraffin diminished the effectiveness of the adjuvants. Subcutaneous and intramuscular injections are followed by a good immunity response, and are to be preferred to intraperitoneal injections.

Shatin and Boyd (1949) attempted to develop Rh antibodies by immunisation of guinea-pigs with human red cells incorporated in adjuvants, but the results were

uniformly disappointing. I am unaware of any reports concerning the use of the Freund-McDermott adjuvants to enhance the development of iso-antibodies to red cells.

## MATERIALS AND METHODS.

*Blood grouping in rabbits.*—Grouping was performed by means of immune sera developed in previous work (Anderson, 1953). These sera determined the presence or absence of two iso-agglutinogens, which for convenience I designated A and B, in the red cells of rabbits. Immune sera were also kindly supplied by Dr. Aaron Kellner of Cornell Medical Center, New York, and it was found that anti-B was identical with Kellner's anti-G, while anti-A was present in Kellner's anti-g (Kellner and Hedal, 1953).

Specimens of blood for grouping were obtained from marginal ear veins and collected into citrate-saline. Since rabbit red cells may show spontaneous agglutination when suspended in physiological saline (Heard, Hinde and Mynors, 1949; Anderson, 1953), the cells were washed with saline and a 3 per cent suspension was prepared in physiological saline containing 5 per cent of normal rabbit serum. Such suspensions showed no spontaneous agglutination and proved suitable for grouping tests. Grouping was also performed on trypsin-treated red cells (*vide infra*).

Blood grouping was done in tubes, the cell suspension being incubated at 37° for 1 hr. with an equal volume of a suitable dilution of grouping serum, and the presence or absence of agglutination was observed with a hand lens. Since individual rabbits show considerable variation in the agglutinability of the red cells by specific iso-agglutinins, the grouping sera were used in low dilutions. It was observed that trypsin-treated cells were strongly agglutinated by high dilutions of grouping sera, and tests employing such cells provided confirmation of the presence or absence of the two agglutinogens, A and B.

*Selection of rabbits.*—Young adult rabbits of mixed breeds were obtained from several dealers. All the rabbits receiving immunising injections were does, while blood for injection was obtained from rabbits of both sexes. The donors and recipients were selected in such a way that each recipient received red cells containing an antigen (either A or B) not present in her own red cells.

*Immunisation procedures.*—Blood from donor rabbits was collected from the marginal ear vein in sterile containers to which sufficient acid-citrate-dextrose (Loutit and Mollison, 1943), or heparin had been added to prevent clotting. The blood was centrifuged, the supernatant fluid removed, and the packed cells were re-suspended in an equal volume of sterile physiological saline. Intravenous injections were given into the marginal ear vein.

Antigen-adjuvant emulsions contained 1 vol. 50 per cent cell suspension in saline, 1 vol. Aquaphor, and 2 vol. liquid paraffin containing 25 mg. of heat-killed dried tubercle bacilli* per 20 ml. Emulsion was effected by means of a glass propellor attached to an air turbine, using sterile precautions throughout.

*Titration of iso-antibodies.*—The titres of iso-antibodies were determined by preparing doubling dilutions of the serum to be tested, and incubating for 1 hr. at 37° with an equal volume of a 3 per cent suspension of suitable red cells. Complete iso-antibodies (saline agglutinins) were titrated by using normal cells suspended in saline containing 5 per cent of normal rabbit serum, while incomplete iso-antibodies were titrated by means of the anti-globulin reaction and also by using red cells treated with trypsin.

The technique of anti-globulin tests was that described previously (Anderson, 1952). Trypsin treatment of red cells was effected by incubating 2 vol. of a 50 per cent red cell suspension in saline for 30 min. at 37° with 1 vol. of a 0·1 per cent solution of crystalline trypsin (Armour) in isotonic phosphate buffer of pH 7·7. The cells were washed thrice with saline both before and after treatment and a 3 per cent suspension was prepared in physiological saline containing 5 per cent normal rabbit serum. Such suspensions sometimes agglutinated weakly when incubated with an equal volume of undiluted or 1/2 dilution of normal rabbit sera, but proved stable in serum diluted more than 1/2, and provided a reliable method for the determination of incomplete iso-antibody.

Trypsin-treated red cells invariably revealed a slightly higher titre of incomplete anti-A and anti-B iso-antibodies than did the anti-globulin reaction, perhaps because the anti-

* I am indebted to Dr. A. E. Francis of the Wellcome Research Laboratories for supplying heat-killed dried tubercle bacilli.

globulin sera, which were developed by immunisation of goats against rabbit serum, were of only moderate potency. A further advantage of the use of trypsin-treated cells was revealed by preliminary tests which indicated that such cells showed little individual variation in agglutinability, the titre of an anti-A or anti-B serum being approximately the same for all specimens of trypsin-treated A-positive or B-positive cells respectively. By contrast, the titre of anti-A sera as determined by titration with normal (untreated) cells, varied considerably with cells from different individuals, an observation which has been made previously by Kellner and Hedal (1953). In the present work, titrations were performed using, for each immune serum, the red cells of the donor which had provided blood for immunising injections, and therefore the titres of immune sera can only be compared when determined with trypsin-treated cells. For this reason, the titres of antibodies recorded in the results refer usually to titrations employing trypsin-treated cells.

In several instances anti-A or anti-B developed along with one or more weaker antibodies, while one rabbit developed a pure antibody which differed in specificity from both anti-A and anti-B, and displayed much higher titres in anti-globulin tests than when tested by trypsin-treated cells ; the recorded titres of this serum are therefore those determined by the former method.

## RESULTS.

Pre-immunisation specimens of serum were obtained from all rabbits which received immunising injections, and, apart from weak agglutinins for trypsin-treated cells (*vide supra*), were uniformly devoid of iso-antibody. Experience showed that titrations performed on specimens of sera obtained 8 days after the termination of each course of immunising injections provided a good indication of the maximal antibody titres attained.

### *First course of immunising injections.*

The first course of injections was similar to that used by Kellner and Hedal (1953). At 48-hr. intervals 23 rabbits each received 20 intravenous injections of red cell suspension ; throughout the course, the volumes of the injections were gradually increased from 1 ml. to 3 ml. Serum was obtained 8 days after the last injection and titrated for iso-antibodies ; the results are given in Table I.

TABLE I.—*Antibody Titres after the First Immunising Course (20 Intravenous Injections).*

| Blood groups of donors and recipients. | Titres of antibodies. | | | |
|---|---|---|---|---|
| | < 4. | 4–16. | 32–256. | 512 or more. |
| 15 A-negative rabbits receiving A-positive R.B.C. | 14 | 1 | *nil* | *nil* |
| 8 B-negative rabbits receiving B-positive R.B.C. | 3 | 3 | 1 | 1 |

The response to this rather prolonged course of injections was disappointing, strong antibody developing in only one rabbit. Accordingly, modification of the immunisation procedure was considered necessary. Previous work (Anderson, 1953) had shown that iso-antibodies could be developed in rabbits by injection of red cells incorporated in Freund-McDermott adjuvants, and that the titres of antibodies so developed could be considerably increased by subsequent administration of a small number of intravenous injections of red cells suspended in saline. This observation was applied in the further immunisation procedures described below.

*Second course of immunising injections.*

After 14 days from the first course of injections, immunisation of the 23 rabbits was recommenced. Section 1, 11 rabbits, received 2 simultaneous injections of 0·5 ml. of red cell-adjuvant emulsion into the subcutaneous tissue of the anterior abdominal wall, followed 7 days later by 3 intravenous injections of 2–3 ml. of 50 per cent red cell suspension at 7-day intervals. The 12 remaining rabbits (Section 2) each received 12 intravenous injections of 2–3 ml. of 50 per cent red cell suspension at 48-hr. intervals. Of 11 normal (previously unimmunised) rabbits 6 were placed in Section 1 and received red cell-adjuvant and intravenous injections, and 5 (with Section 2) received intravenous injections alone. Thus each section contained 17 rabbits which were selected in such a way that those which had previously developed antibodies were divided equally between the two sections. Antibody titrations were again performed 8 days after the last injections, and the results are summarised in Table II.

Although iso-antibodies developed in rabbits in both sections, the titres were, in general, far higher in those in Section 1, which received red cell-adjuvant emulsions; 8 of these rabbits developed antibody of titre 256 or more, and in 5 of these the titre was as high as 2048, whereas only one rabbit of Section 2, which received intravenous injections alone, developed antibody of titre greater than 128. This contrast in antibody titres in rabbits of the two sections is not attributable to differences in antigenicity of the donors' red cells, since the red cells of each donor were injected into one or more recipients in each section.

*Third course of immunising injections.*

The 9 rabbits in Section 1, and 15 rabbits in Section 2, which developed either no antibody or antibody of titre 64 or less, received 2 simultaneous subcutaneous injections of 0·5 ml. of red cell-adjuvant emulsion followed 1 week later by 3 intravenous injections of 2–3 ml. of red cell suspension at weekly intervals. At 8 days from the last injection, the sera of these recipients were again titrated, and it was found that 3 of the 9 Section 1 rabbits and 6 of the 15 Section 2 rabbits had developed strong iso-antibodies (Table III).

## DISCUSSION.

As already stated, the immunisation procedures described above were undertaken in order to develop immune iso-antibodies of high titre, and not primarily to compare two methods of iso-immunisation. Nevertheless, it is considered that the results do indicate the superior antibody response following injection of adjuvants. This conclusion is based largely on the titres of antibodies which resulted from the second course of injections (Table II). Here, the efficacy of red cell-adjuvant injections followed by 3 intravenous injections of red cells in saline may be compared with that of a course of 12 intravenous injections of red cells in saline. Of 17 rabbits (Section 1) receiving the former, 7 developed strong iso-antibodies, while none of the 17 rabbits (Section 2) which received the latter course developed strong iso-antibodies.

The injection courses which did not employ adjuvants involved the administration of 644 intravenous injections to 28 rabbits (first course and second course, Section 2), only one of which developed strong iso-antibody. By contrast, the administration of 41 (each divided into 2 simultaneous injections) subcutaneous

TABLE II.—*Antibody Titres after the Second Course of Immunising Injections.*

| | | Section 1 (adjuvant emulsion and 3 intravenous injections). | Section 2 (12 intravenous injections alone). |
|---|---|---|---|
| Total number of recipients | | 17 | 17 |
| Blood groups of donors and recipients : | Total number | 11 | 12 |
| | Antibody titres : | | |
| A-negative rabbits receiving A-positive R.B.C. | < 4 | 6 | 9 |
| | 4–16 | *nil* | 1 |
| | 32–128 | 1 | 2 |
| | 256–1024 | 2 | *nil* |
| | 2048 or more | 2 | *nil* |
| | Total number | 6 | 5 |
| | Antibody titres : | | |
| B-negative rabbits receiving B-positive R.B.C. | < 4 | *nil* | 1 |
| | 4–16 | 1 | *nil* |
| | 32–128 | 1 | 3 |
| | 256–1024 | 1 | 1 |
| | 2048 or more | 3 | *nil* |

TABLE III.—*Antibody Titres after the Third Course of Immunising Injections.*

| | | Section 1. | Section 2. |
|---|---|---|---|
| Total number of recipients | | 9 | 15 |
| Blood groups of donors and recipients : | Total number | 7 | 11 |
| | Antibody titres : | | |
| A-negative rabbits receiving A-positive R.B.C. | < 4 | 4 | 5 |
| | 4–16 | 0 | 1 |
| | 32–256 | 1 | 2 |
| | 512 or more | 2 | 3 |
| | Total number | 2 | 4* |
| | Antibody titres : | | |
| B-negative rabbits receiving B-positive R.B.C. | < 4 | *nil* | *nil* |
| | 4–16 | 1 | *nil* |
| | 32–256 | *nil* | *nil* |
| | 512 or more | 1 | 3 |

* One rabbit in this group died of an acute respiratory infection.

injections of red cell-adjuvant emulsion followed by a total of 123 intravenous injections of red cells in saline, to 32 rabbits (second course, Section 1 and third course), resulted in strong iso-antibodies in 16 instances. The use of adjuvants reduces not only the number of injections required to stimulate antibody development, but also the duration of the immunisation course.

The question arises whether it is necessary to incorporate red cells in the adjuvant emulsion in order to obtain enhanced immunity or whether the injection of the oily suspension of tubercle bacilli brings about a condition in which the rabbit

responds more strongly to subsequent intravenous injections of red cells. Preliminary investigations suggest that the latter hypothesis is correct, since it was observed that rabbits receiving injections of adjuvant emulsion containing red cells of their own ABO blood group responded well to subsequent intravenous injections of red cells of a different group. Moreover, Cohen (1954) observed that rabbits which had received injections of B.C.G. were more readily iso-immunised by intravenous injections of red cells than were normal rabbits, a finding in agreement with the above conclusion.

SUMMARY.

Red cells incorporated in Freund-McDermott adjuvant emulsions were injected subcutaneously into rabbits, and followed by 3 intravenous injections of red cells in saline. This procedure is less tedious and time-consuming than immunisation by multiple intravenous injections of red cells, and is more successful in developing iso-antibodies of high titre.

This investigation was carried out in the Department of Pathology, University of Rochester School of Medicine and Dentistry, Rochester, N.Y., during the tenure of a Rockefeller Travelling Fellowship in Medicine, 1953–54. I wish to thank Dr. G. H. Whipple for providing facilities.

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# THE RÔLE OF BACTERIA IN THE PATHOGENESIS OF HEPATIC ARTERIAL INFARCTION IN THE RABBIT.

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IT has been claimed (Dujarier and Castaigne, 1899; Markowitz, Rappaport and Scott, 1949; Tanturi, Swigart and Canepa, 1950; Chau, Goldbloom and Gurd, 1951) that in the dog ligation of the hepatic artery produces a state of relative anoxia in the liver, as a result of which anaerobic organisms of the genus *Clostridium* flourish and form toxins which are largely responsible for the necrosis seen in the liver and for the death of the animal. Such bacterial growth can, it is claimed, be prevented by the administration of penicillin, and the prophylactic use of this antibiotic has therefore been advocated in operations during which the hepatic artery is exposed to accidental or intentional ligation. In our experience, however, there is no indication that proliferation of clostridia occurs after occlusion of the hepatic artery in man. Nor have we found in the literature any good evidence that it is a feature of hepatic infarction in animals other than the dog. In an attempt to confirm this apparent species difference we have therefore repeated in part the detailed experiments performed by Cameron and Mayes (1930) on rabbits, modifying these authors' procedure in order to investigate the bacterial flora of the liver after ligation of the hepatic artery both with and without the administration of penicillin.

## MATERIAL AND METHODS.

Twelve adult rabbits were used, 10 males and 2 females. They were kept on a normal laboratory diet of green vegetables and carrots throughout. They were anaesthetised with ether, their abdominal walls were shaved and they were placed upon their backs with a cloth pad beneath the upper abdomen as recommended by Cameron and Mayes. The operation site was then sterilised and the abdomen was opened by a median epigastric incision under strictly aseptic conditions. Bleeding was minimal and bleeding points rarely required more than temporary occlusion with Spencer Wells forceps. The hepatic artery was exposed in the porta hepatis by gentle displacement of the liver and stomach, ligated twice with cotton thread and then divided between the ligatures. In every case the artery was ligated at a point distal to the origin of the branch to the right lateral lobe of the liver (the point $\gamma$ in Cameron and Mayes' diagram). The right lateral lobe therefore (and usually the omental lobe as well) escaped infarction. The abdomen was then closed and a dressing applied.

In 6 animals penicillin was administered as follows. Half an hour before the operation 100,000 units of soluble penicillin were injected intramuscularly. On the conclusion of the operation and at 24-hr. intervals thereafter until the animal was killed 75,000 units of procaine penicillin and 25,000 units of soluble penicillin were injected by the same route.

All the animals survived the operation; they were killed at varying intervals after it by means of carbon monoxide gas. In all but two the livers were removed with aseptic precautions and examined bacteriologically as follows. Swabs were taken from the lesions and plated on blood 2 per cent agar and MacConkey's medium to be incubated aerobically, and on blood 6 per cent agar to be incubated anaerobically in a McIntosh and Fildes' jar. In addition a tube of serum broth was incubated aerobically and a portion of liver (usually

about 1 ml. in volume) was incubated anaerobically in serum broth. Subcultures were made when necessary. After these cultures had been made blocks of tissue were removed for histological examination in the usual manner. Paraffin sections were stained by haematoxylin and eosin, and in many cases by Gram's and other methods.

## RESULTS.

In order to assess the effects of penicillin in preventing hepatic infarction the animals were not killed before 48 hr. so that the lesions might be well established and readily recognisable. Six animals killed after intervals of 48–240 hr. which had received no penicillin were therefore compared with six animals killed between 48 and 168 hr. which had received penicillin in the dosages described above. The results are shown in the Table. It will be seen that penicillin had no appreciable effect on the results of hepatic artery ligation. In none of the animals receiving penicillin was infarction prevented, and while in two the lesions were small, in the remaining four they were as extensive as in the control animals.

TABLE.—*Occlusion of Hepatic Artery without Penicillin.*

| Rabbit. | Time killed. | Presence of infarction. | Bacteriology. |
|---|---|---|---|
| 1 | 48 hr. | Moderately extensive infarction | — |
| 2 | 48 „ | Single infarct with a few flecks of necrosis elsewhere | Aerobic: *Bact. coli, Proteus, Pseudomonas.* Anaerobic: sterile. |
| 3 | 67 „ | Moderately extensive infarction | — |
| 4 | 90 „ | Very extensive infarction | Aerobic: *Staph. albus.* Anaerobic: sterile. |
| 5 | 120 „ | Extensive infarction | Aerobic: sterile. Anaerobic: „ |
| 6 | 240 „ | Very extensive infarction | Aerobic: sterile. Anaerobic: „ |
| | | *Occlusion of Hepatic Artery with Penicillin.* | |
| 7 | 48 „ | Small infarct only. Necrosis in gall bladder bed. | Aerobic: sterile. Anaerobic: „ |
| 8 | 91 „ | Extensive infarction | Aerobic: sterile. Anaerobic: „ |
| 9 | 96 „ | „ „ | Aerobic: sterile. Anaerobic: „ |
| 10 | 120 „ | Extensive zonal necrosis. Small infarct only. | Aerobic: sterile. Anaerobic: „ |
| 11 | 146 „ | Extensive infarction | Aerobic: sterile. Anaerobic: „ |
| 12 | 168 „ | „ „ | Aerobic: sterile. Anaerobic: „ |

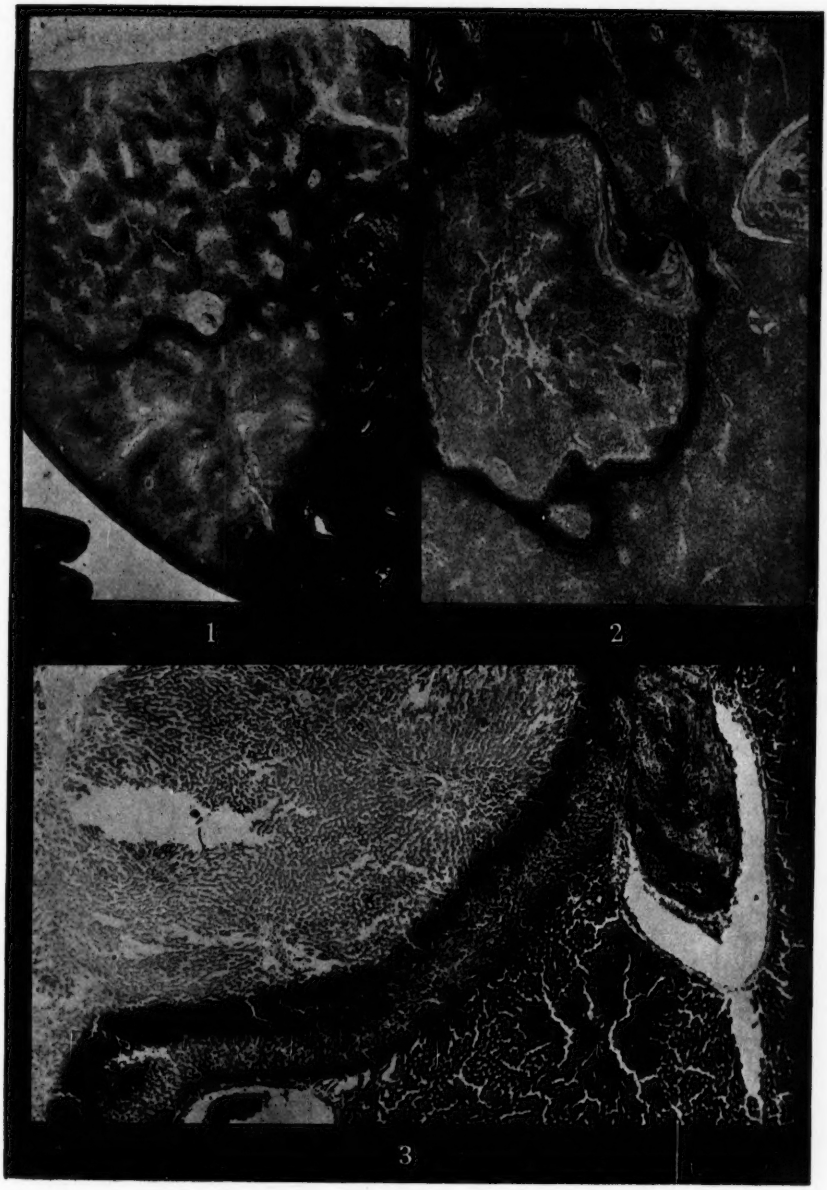
— = cultures not made.

## DESCRIPTION OF PLATES.

Fig. 1 (Rabbit 9). The infarct occupies all but the lower right part of the field. That part of it below is more recent than the remainder, from which it is separated by a narrow band of nuclear débris (the remains of a zone of polymorphonuclear infiltration). Haematoxylin and eosin. × 9.

Fig. 2 (Rabbit 6). The only surviving tissue is in the left upper corner of the field. The infarct is divided into two areas differing in age and separated by a band of nuclear débris. Haematoxylin and eosin. × 12.

Fig. 3 (Rabbit 2). To the left and above is completely necrotic tissue, to the right and below normal liver. Between the two is a narrow deeply haematoxyphil band of polymorphonuclear infiltration and a broader band of parenchymal necrosis. Haematoxylin and eosin. × 35.



Parker and Campbell.

Cultivation of the lesions also lent no support to the view that bacterial growth is of importance in the pathogenesis of hepatic infarction in the rabbit. In all but two of the animals the lesions were sterile and in none were clostridia, the organisms incriminated by previous workers, grown; the growth in the positive cultures from rabbits 2 and 4 was scanty and probably due to contamination. Clinically, too, there was no evidence of bacterial toxaemia or of septicaemia. The animals recovered rapidly from the operation and were soon eating normally.

The morphology of hepatic arterial infarction in the rabbit has been described in detail by Cameron and Mayes (1930) and it is only necessary therefore to make a few points here. Firstly, no differences were noted between the lesions of animals receiving penicillin and those of the controls. Secondly, the necrosis took the form of localised infarcts and did not involve the entire liver, as is apparently sometimes the case in the dog. There was, however, evidence that in some cases at least the infarction was progressive. With only one striking exception (rabbit 10) the animals killed at 90 hr. and afterwards had considerably more extensive lesions than those sacrificed earlier. Rabbit 6, killed at 10 days, had the most extensive lesions of all, very little liver tissue surviving in the lobes deprived of their arterial blood supply. Microscopically there was good evidence of progression in rabbits 5, 6, 9 and 12. In these animals there were necrotic areas of different ages separated from one another by what had previously been a peripheral zone of leucocytic infiltration (Fig. 1 and 2). Cameron and Mayes also recorded that necrosis might extend slowly through the liver. We have not seen this feature in the corresponding human lesions. Finally a further point of difference between arterial infarcts in the rabbit and in man may be noted. In our experience there is in the great majority of human infarcts a broad peripheral zone in which the hepatic stroma survives but in which the parenchyma shows a greater or less degree of centrilobular zonal necrosis. In our experimental lesions, on the other hand, the corresponding peripheral zone of parenchymal but not stromal necrosis was very narrow, being rarely more than half a lobule wide (Fig. 3). Only in one animal (rabbit 10) did the appearances in this respect resemble those seen in man.

## DISCUSSION.

From the results of this experiment it may be concluded that the rabbit and the dog exhibit important differences in their reaction to ligation of the hepatic artery. It would seem probable that these differences are related to differences in the bacterial flora of the normal tissues in these species. The presence in the normal dog's liver of anaerobic organisms of the genus *Clostridium* has been reported by Wolbach and Saki (1909), Ellis and Dragstedt (1930) and Trusler and Reeves (1934); the relation of these organisms to the fatal results which follow introduction of liver tissue into the peritoneal cavity of the dog is reviewed by Markowitz and Rappaport (1951). These last authors maintain that ligation of the hepatic artery in the dog results in a relative anoxia within the liver which allows the clostridia normally present to flourish.

In the rabbit's liver, on the other hand, we believe that clostridia are not usually present and play no essential part in the production of the necrosis which follows arterial ligation. Because of this species difference between the dog and the rabbit we consider it unwise to apply uncritically to a third species, namely man, experimental results obtained in either of the first two. None the less, a

study of human hepatic infarction has suggested strongly to us that in his response to arterial ligation man resembles the rabbit more nearly than he does the dog. Despite the minor differences noted above (which presumably reflect minor differences in the hepatic vasculature) arterial infarcts in man resemble closely those seen in the rabbit. In particular the gas gangrene type of lesion described in the dog is not seen. This aspect of the problem will be developed in a further communication.

SUMMARY.

An experiment is described which was designed to assess the rôle of anaerobic bacteria in the production of liver necrosis following ligation of the hepatic artery in the rabbit.

Of 12 rabbits in which the hepatic artery was ligated 6 were given penicillin pre- and post-operatively. There was no difference between the incidence of infarction in these animals and in the control group. Anaerobic bacteria were not isolated from any animal, and the majority of lesions were sterile.

It is concluded that in this respect there is a species difference between the dog and the rabbit, for in the former a type of gas gangrene is reported to follow ligation of the hepatic artery.

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# SOME FACTORS INFLUENCING THE RESPONSE TO IMMUNISATION WITH SINGLE AND COMBINED PROPHYLACTICS.

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EARLIER work (Barr and Llewellyn-Jones, 1953*a*, *b*) has shown that interference with the development of immunity to tetanus may occur in animals immunised with two injections of a combined prophylactic containing another antigen to which they already have some immunity. Similar interference occurred when the first injection was of the combined prophylactic and the second injection of tetanus toxoid alone, but not when the order of the injections was reversed. These results were obtained in diphtheria-immune guinea-pigs subsequently immunised with D.T.P. (diphtheria-tetanus prophylactic) and in T.A.B.-immune guinea-pigs immunised with T.A.B.T. (typhoid-paratyphoid A and B vaccine + tetanus toxoid). The interference with the response to tetanus toxoid, observed in groups receiving a combined prophylactic for the first dose, must have been due to an effect of the secondary response to the other component of the prophylactic.

Further experiments have now been done in an attempt to determine the circumstances in which interference occurs.

## EXPERIMENTAL.

### *The Effect of Varying the Interval Between Injections of Combined Prophylactic.*

Normal guinea-pigs were injected with a single dose (0·3 Lf) of a diphtheria prophylactic consisting of purified diphtheria toxoid adsorbed on aluminium hydroxide. In this and all other experiments, materials were suitably diluted in normal saline and injected subcutaneously in a dose of 1 ml. The animals were rested for three months, at the end of which time they were re-weighed. Normal guinea-pigs of comparable weight were selected as controls, and all received a dose of 0·005 ml. of diphtheria-tetanus prophylactic (D.T.P.) containing, per ml., 60 Lf of purified diphtheria toxoid and 7 Lf of purified tetanus toxoid adsorbed on aluminium hydroxide. The guinea-pigs were further subdivided and received a second dose of D.T.P. after different intervals of time, as shown in Table I, which gives the tetanus antitoxin titres of sera taken from the animals 10 days after the second injection. The sera were titrated by the mouse method described by Glenny and Stevens (1938). The numbers were small, but the figures in Table I suggest that both the diphtheria-immune and the normal control animals profited by delaying the second dose from 4 to 8 weeks, but that both groups gave a poorer response when the interval between injections was increased to 12 weeks. This result suggests that the tetanus toxoid injected was insufficient for the establishment of adequate basal immunity for longer than about 8 weeks after the first dose. The work was therefore repeated using a different prophylactic which

contained the same amount of diphtheria toxoid but approximately double the amount of tetanus toxoid. Table II shows the results of this experiment, in which the range of interval between injections was extended, and the results for each group except for 24-week ones consist of the sum of the figures obtained from two or more groups of 10–20 animals injected on different occasions.

It is clear from Table II that there was no significant difference between the tetanus antitoxin responses of the various groups of normal controls 10 days after the second injection of D.T.P. Some interference with the tetanus response occurred in all groups which had received an earlier injection of diphtheria prophylactic, but this was minimal in the animals which received the two doses of D.T.P. separated by an interval of 12 weeks. The distribution of antitoxin titres in these groups is interesting. In the 12-week group it approximated to the normal distribution, but the peak and the geometric mean (9·92) were lower than in the corresponding controls. This suggests that the pre-existing diphtheria immunity caused interference with the tetanus response of the animals in the group as a whole, but was insufficient to produce a sharp division between good and poor responders. In all other groups the peak of the main distribution was at a lower titre than that of the corresponding controls, but there was, in addition, a break in the distribution, so that the very poor responders of the groups became separated from the remainder. It may appear, on inspection of the figures in Table II, that the tetanus antitoxin titres of diphtheria-immune animals receiving the two injections of D.T.P. separated by an interval of 24 weeks were higher than in those receiving them separated by an interval of 16 weeks. It will, however, be noted that the number of animals in the latter group was greater, and that the peak of the distribution occurred at a higher antitoxin level than that for the guinea-pigs which received the two injections separated by an interval of 24 weeks.

The decrease in the degree of interference, brought about by lengthening the interval between the two injections of D.T.P. to 12 weeks, suggests that a prolonged but decreasing crowding-out effect was produced by the secondary response to diphtheria toxoid in the *first* dose of D.T.P. on the secondary response to tetanus toxoid in the *second* dose of D.T.P. The duration of the secondary diphtheria response effect cannot, however, completely account for the interference with the response to the course of immunisation with the tetanus toxoid in the combined prophylactic. If this were the sole cause, the effect of further increase, beyond 12 weeks, of the interval between injections would be finally to abolish any interference. It therefore seems probable that, in addition, the speed of the secondary response to the diphtheria toxoid in the first dose of D.T.P. brought about a crowding-out of the development of basal immunity to the tetanus toxoid present in the first dose. It is even possible that this latter phenomenon was the sole cause of the interference, but it will be shown later in this paper that antibody production following secondary stimulation may continue for at least 28 days.

An exactly similar experiment was carried out in which T.A.B.-immune and normal control guinea-pigs were immunised with two doses, each of 0·05 ml. of T.A.B.T. injected at varying intervals of time. The tetanus antitoxin titres, measured 10 days after the second injection, are shown in Table III. The results are substantially the same as those shown in Table II for diphtheria-immune animals immunised with D.T.P. On increasing the interval between injections, there was first an improvement and then a relapse in the responses of the T.A.B.-immune guinea-pigs. In this experiment, the optimal interval was 8 weeks. The

TABLE I.—*The Tetanus Antitoxin Responses of Diphtheria-immune and Normal Guinea-pigs Immunised with Two Doses of Diphtheria-tetanus Prophylactic (D.T.P.) Separated by Varying Intervals of Time.*

| Interval between injections of D.T.P. | Group. | Number of guinea-pigs with tetanus antitoxin titres (units/ml.). > / < 0·01 | 0·01 0·02 | 0·02 0·05 | 0·05 0·1 | 0·1 0·2 | 0·2 0·5 | 0·5 1·0 | 1·0 2·0 | 2·0 5·0 | 5·0 10 | 10 20 | 20 50 | 50 100 | Total. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 weeks | Diph.-immune | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 6 | 2 | 1 | — | — | 13 |
| | Normal | — | — | — | — | — | — | — | — | 1 | 3 | 4 | 3 | — | 11 |
| 8 „ | Diph.-immune | — | — | — | — | — | — | — | 5 | 2 | 5 | 2 | — | — | 14 |
| | Normal | — | — | — | — | — | — | — | — | — | 1 | 5 | 4 | 1 | 11 |
| 12 „ | Diph.-immune | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 5 | 3 | 0 | 1 | — | 13 |
| | Normal | — | — | — | — | — | — | — | — | 2 | 4 | 7 | 1 | — | 14 |

The prophylactic contained, per ml., 60 Lf of purified diphtheria toxoid and 7 Lf of purified tetanus toxoid.

TABLE II.—*Tetanus Antitoxin Responses of Diphtheria-immune and Normal Guinea-pigs Immunised with Two Doses of Diphtheria-tetanus Prophylactic (D.T.P.) Separated by Varying Intervals of Time.*

| Interval between injections of D.T.P. | Group. | Number of guinea-pigs with tetanus antitoxin titres (units/ml.). > / < 0·01 | 0·01 0·02 | 0·02 0·05 | 0·05 0 1 | 0·1 0·2 | 0·2 0·5 | 0·5 1·0 | 1·0 2·0 | 2·0 5·0 | 5·0 10 | 10 20 | 20 50 | 50 100 | 100 200 | Total. | Geometric mean. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 weeks | Diph.-immune | — | — | — | 1 | 0 | 2 | 0 | 2 | 6 | 7 | 5 | 1 | — | — | 24 | 3·96 |
| | Normal | — | — | — | — | — | — | — | — | 1 | 3 | 10 | 10 | 3 | 1 | 28 | 21·4 |
| 8 „ | Diph.-immune | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 6 | 5 | 11 | 13 | 4 | — | — | 42 | <5·5 |
| | Normal | — | — | — | — | — | — | — | — | — | 5 | 7 | 35 | 6 | — | 53 | 27·0 |
| 12 „ | Diph.-immune | — | — | — | — | — | — | — | 4 | 4 | 13 | 15 | 10 | — | — | 46 | 9·92 |
| | Normal | — | — | — | — | — | — | — | — | — | 3 | 13 | 24 | 3 | — | 43 | 23·6 |
| 16 „ | Diph.-immune | — | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 4 | 4 | 12 | 7 | — | — | 31 | 7·27 |
| | Normal | — | — | — | — | — | — | — | — | — | 3 | 10 | 15 | 2 | — | 30 | 22·0 |
| 24 „ | Diph.-immune | — | — | — | — | — | 1 | 1 | 1 | 0 | 7 | 7 | 2 | — | — | 19 | 7·39 |
| | Normal | — | — | — | — | — | — | — | — | — | 2 | 7 | 6 | — | — | 15 | 17·8 |

The prophylactic contained, per ml., 60 Lf purified diphtheria toxoid and 13 Lf of tetanus toxoid.

TABLE III.—*Tetanus Antitoxin Responses of T.A.B.-immune and Normal Guinea-pigs Immunised with Two Doses of T.A.B.T. Separated by Varying Intervals of Time.*

| Interval between injections of T.A.B.T. | Group. | Number of guinea-pigs with tetanus antitoxin titres (units/ml.). | | | | | | | | | | | | | | Total | Geometric mean. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | <0·01 | 0·01–0·02 | 0·02–0·05 | 0·05–0·1 | 0·1–0·2 | 0·2–0·5 | 0·5–1·0 | 1·0–2·0 | 2·0–5·0 | 5·0–10 | 10–20 | 20–50 | 50–100 | 100–200 | | |
| 4 weeks | T.A.B.-immune | 2 | 0 | 0 | 0 | 2 | 1 | 1 | 3 | 9 | 7 | — | — | — | — | 25 | <1·56 |
| | Normal | — | — | — | — | — | — | — | 1 | 11 | 14 | 7 | 7 | — | — | 40 | 7·99 |
| 6 ,, | T.A.B.-immune | — | — | — | — | — | — | 3 | 6 | 2 | 1 | — | — | — | — | 12 | 1·56 |
| | Normal | — | — | — | — | — | — | — | — | 4 | 7 | 11 | 1 | 1 | — | 24 | 9·95 |
| 8 ,, | T.A.B.-immune | — | — | — | — | — | — | — | — | 2 | 5 | 4 | — | — | — | 11 | 7·86 |
| | Normal | — | — | — | — | — | — | — | 1 | 3 | 5 | 3 | 7 | 1 | — | 20 | 12·2 |
| 12 ,, | T.A.B.-immune | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 4 | 6 | 1 | 2 | — | — | 17 | <3·2 |
| | Normal | — | — | — | — | — | — | — | 2 | 1 | 5 | 5 | 3 | 2 | 1 | 19 | 13·0 |
| 16 ,, | T.A.B.-immune | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 4 | 2 | — | — | — | 13 | <2·8 |
| | Normal | — | — | — | — | — | — | 1 | 0 | 1 | 3 | 3 | 5 | 0 | 1 | 14 | 13·9 |

The prophylactic contained, per ml., $10^9$ *S. typhi*, $5 \times 10^8$ each *S. paratyphi* A and B and 8 Lf of tetanus toxoid.

mean of the titres of the normal control animals showed a progressive but not significant increase as the interval between injections was increased up to 8 weeks. Thereafter there was no increase, but the responses of the T.A.B.-immune animals were poorer.

The least interference occurred when the interval between injections of the diphtheria-immune guinea-pigs immunised with D.T.P. was 12 weeks: for the T.A.B.-immune guinea-pigs immunised with T.A.B.T. interference was least when the interval was 8 weeks. This difference may be due to difference in dosage and composition of the prophylactics and the degree of potential immunity established by them, or to differences in the immunological effects of a secondary response to a toxoid and to a bacterial vaccine.

*The Effect of Primary Stimulation with One Antigen on Responses to a Subsequent Course of Immunisation with Another Antigen.*

In this experiment normal guinea-pigs were injected with a single dose of 0·05 ml. of T.A.B. vaccine containing per ml. $10^9$ *Salmonella typhi* and $5 \times 10^8$ each *Salmonella paratyphi* A and B, at varying intervals of time before the first of two injections of tetanus toxoid separated by an interval of 2 months. The animals were bled 10 days after the second injection and the sera titrated for tetanus antitoxin content. The results are shown in Table IV.

TABLE IV.—*Effect on the Tetanus Antitoxin Response of a Single Dose of T.A.B. Vaccine given at Varying Times before the First of Two Injections of Tetanus Toxoid Separated by an Interval of 2 Months.*

| Interval between T.A.B. injection and first dose of tetanus toxoid. | Number of guinea-pigs with tetanus antitoxin titres (units/ml.) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | > <0·01 | 0·01 0·02 | 0·02 0·05 | 0·05 0·10 | 0·10 0·20 | 0·20 0·50 | 0·50 1·0 | 1·0 2·0 | 2·0 5·0 | 5·0 10 | 10 20 | 20 50 | 50 100 | Total. |
| 7 days | 3 | — | — | — | — | — | — | 1 | 3 | 7 | 1 | — | — | 15 |
| 14 ,, | — | — | — | — | — | 2 | — | — | 2 | 2 | 7 | 2 | — | 15 |
| 21 ,, | — | — | — | — | — | — | — | 1 | 1 | 4 | 4 | 4 | 1 | 15 |
| 28 ,, | — | — | — | — | — | — | — | 4 | 4 | 6 | 8 | 5 | 3 | 30 |
| Control (no preliminary T.A.B.) | — | — | — | — | — | — | 1 | 4 | 5 | 9 | 6 | 3 | 1 | 29 |

It will be seen that interference with the response to immunisation with tetanus toxoid occurred only in those groups of animals which had been primarily stimulated with T.A.B. vaccine 7 or 14 days previously. The primary stimulation had no effect on the results of tetanus toxoid immunisation if this were delayed for 21 days or longer after the dose of vaccine.

A similar experiment was carried out, in which groups of normal guinea-pigs received a moderately large dose (1 Lf) of diphtheria prophylactic at various intervals of time before the first of two injections of purified tetanus toxoid adsorbed on aluminium hydroxide. The diphtheria prophylactic used consisted also of purified toxoid adsorbed on aluminium hydroxide. The guinea-pigs were bled 10 days after the second injection of tetanus prophylactic and the antitoxin titres are shown in Table V.

TABLE V.—*Effect on the Tetanus Antitoxin Response of a Single Dose of Diphtheria Prophylactic, Given at Varying Times before the First of Two Injections of Tetanus Toxoid, Separated by an Interval of 28 Days.*

| Interval between diphtheria injection and first dose of tetanus toxoid | Number of guinea-pigs with tetanus antitoxin titres (units/ml.) > / < 0·01 | 0·01 0·02 | 0·02 0·05 | 0·05 0·10 | 0·10 0·20 | 0·20 0·50 | 0·50 1·0 | 1·0 2·0 | 2·0 5·0 | 5·0 10 | 10 20 | 20 50 | Total. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 days | | 6 | 0 | 0 | 1 | 2 | 2 | 4 | 7 | 3 | 0 | 1 | 26 |
| 7 ,, | | 8 | 1 | 1 | 1 | 0 | 2 | 1 | 6 | 3 | 2 | 1 | 1 | 27 |
| 14 ,, | | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 6 | 7 | 6 | 6 | 1 | 29 |
| 21 ,, | | 4 | 0 | 0 | 0 | 0 | 2 | 0 | 8 | 7 | 7 | 1 | — | 29 |
| 28 ,, | | — | — | — | — | — | — | — | 5 | 6 | 7 | 9 | — | 27 |
| Control (no preliminary diphtheria injection) | | — | — | — | — | — | — | — | — | — | 5 | 7 | 3 | 15 |

It is evident that very considerable interference occurrred with the response to tetanus immunisation, and that this interference was greatest in the group which received the first dose of prophylactic 7 days after the single dose of diphtheria prophylactic. Considerable interference occurred when the first dose was given as early as 3 days after the primary stimulation with diphtheria prophylactic, and as late as 28 days the responses, though much improved, were significantly lower than those of the control group ($P = \leqslant 0{\cdot}01$).

This interference cannot be associated with the time of appearance of diphtheria antitoxin in the blood. If this were the explanation, no interference would be expected to occur in guinea-pigs receiving the first dose of tetanus prophylactic 3, 7 and possibly also 14 days after the single dose of diphtheria prophylactic. The results of experiments of this kind might well be expected to depend on the type of prophylactic used for the "non-specific" primary stimulus (whether fluid or adsorbed), the dosage and the proximity of the sites of injection of the two antigens. In this work all injections were made subcutaneously through the abdominal wall.

*The Effect of Secondary Stimulation with One Antigen on Responses to a Subsequent Course of Immunisation with Another Antigen.*

Normal guinea-pigs received two doses of diphtheria prophylactic separated by an interval of 28 days. At varying times after the second dose, the first of two doses of tetanus toxoid was injected: the second dose was given 28 days after the first, and the guinea-pigs were bled 10 days later.

Table VI gives the tetanus antitoxin titres of the groups of guinea-pigs. Fifteen diphtheria-immune animals were injected in each experiment, but the control experiment was performed on two occasions, using a total of 29 animals.

Interference with the response to tetanus toxoid occurred in all groups, but was least in those which received the first dose 3 days after secondary stimulation with diphtheria toxoid. In this group all the animals produced detectable antitoxin, but the scatter of titres was large. When the interval between the second dose of diphtheria toxoid and the first dose of tetanus toxoid was increased, some animals failed to produce 0·01 unit of antitoxin (the lowest level tested) even when the interval was as long as 28 days. It would therefore appear that antibody-forming activity concerned in crowding-out responses is of longer duration after secondary stimulation than after primary stimulation, but is initiated slightly later.

TABLE VI.—*Effect on the Tetanus Response of Secondary Stimulation with Diphtheria Prophylactic, at Varying Times before the First of Two Injections of Tetanus Prophylactic Separated by an Interval of 28 Days.*

| Interval between diphtheria injection and first dose of tetanus toxoid. | Number of guinea-pigs with tetanus antitoxin titres (units/ml.). | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | > <0·01 | 0·01 0·02 | 0·02 0·05 | 0·05 0·10 | 0·10 0·20 | 0·20 0·50 | 0·50 1·0 | 1·0 2·0 | 2·0 5·0 | 5·0 10 | 10 20 | 20 50 | Total. |
| 3 days | — | — | — | — | — | 1 | 2 | 1 | 2 | 6 | 1 | 2 | 15 |
| 7 „ | 2 | — | — | — | — | — | — | 2 | 5 | 3 | 3 | — | 15 |
| 10 „ | 3 | — | — | — | — | — | — | 1 | 4 | 3 | 4 | — | 15 |
| 14 „ | 3 | — | — | 1 | — | — | — | 2 | 4 | 2 | 3 | — | 15 |
| 21 „ | 4 | — | 1 | — | — | — | 1 | 1 | 4 | 2 | 2 | — | 15 |
| 28 „ | 1 | 1 | — | — | — | — | 1 | 5 | 3 | 2 | 1 | — | 14 |
| Control (no preliminary diphtheria injection) | — | — | — | — | — | — | — | 3 | 11 | 8 | 7 | — | 29 |

## DISCUSSION.

These experiments confirm our earlier observation that there may be danger in using a combined prophylactic for the purpose of simultaneously boosting the titre of one antibody and producing another in primary immunisation. The mechanism of interference with the primary immunisation by the secondary response of the animal to the other antigen remains obscure. There is evidence from the data in Tables II and III that the speed of the secondary response to the antigen to which there was pre-existing immunity brought about crowding-out of the development of basal immunity to tetanus toxoid. In addition, results in Table VI show that prolonged antibody-forming activity follows secondary stimulation with toxoid ; this may have interfered with the response to the tetanus toxoid in the second injection of combined prophylactic (Tables II and III).

Other results (Tables IV and V) show that unsatisfactory responses may follow courses of immunisation in which the first dose of one course is given at too short an interval before the first dose of a second course. The primary response to the first may crowd out the development of basal immunity to the second. In our experiments interference with the response to immunisation with one antigen occurred when the first dose was given as early as 3 days after primary stimulation with another antigen. This is long before any antitoxin becomes detectable in the circulation, and indeed the interference had already diminished when the first dose of the course was delayed until 14–21 days after the non-specific primary stimulation ; it is at this time that antitoxin is demonstrable in the circulation of most animals.

It also appears dangerous to start a course of immunisation with one antigen until some weeks have elapsed since the completion of a course in which another antigen was used. The prolonged activity involved in the secondary response to the final injection of the first course may interfere with the development of basal immunity to the first dose of the second course. The results suggest that antigenic stimulation caused by subclinical or clinical infections might cause similar interference with the response to artificial immunisation with prophylactics, if this were started before the effects of the natural stimulation had died down.

If these results are directly applicable to human immunisation, injections of prophylactic should not be given without making sure that no other injection of antigen, whether a primary or secondary stimulus, has been given for at least a

month previously. The facts should also be borne in mind in devising programmes of immunisation of babies involving smallpox vaccination and separate courses of immunisation with diphtheria and tetanus prophylactics and *Haemophilus pertussis* vaccine.

The duration of antibody-forming activity following primary and secondary stimulation may well depend on the type of prophylactic and the dosage used. It is possible that the marked interference with the response to immunisation shown in Table V would have been less if the dose of diphtheria prophylactic given previously had been smaller, or if the dose had been administered as fluid toxoid with no mineral carrier. It is also possible that such interference might be reduced if the second antigen were injected into a different site from the first, and if animals with immunity to one component of a combined prophylactic were immunised, not with the combined prophylactic as such, but with the component antigens administered in widely separated sites. Much work therefore remains to be done. It is possible that in later work some light may be thrown on the more fundamental processes of interference with antibody production.

SUMMARY.

Interference with the response to two injections of tetanus toxoid occurred in guinea-pigs immunised with two injections, separated by different intervals of time, of a combined prophylactic containing another antigen to which they already had immunity. As the interval between injections was increased, the interference became less but eventually greater again. It is suggested that activity connected with the secondary response of the animals to the other antigen, present in the first dose of combined prophylactic, caused crowding-out of the response to the tetanus toxoid in the first and second doses.

A primary stimulus of diphtheria prophylactic suppressed the response to a course of two injections of tetanus toxoid when the first of these was given at times ranging from 3 to 21 days later; when the interval was 28 days interference, though significant, was reduced. The greatest interference occurred when the first dose of tetanus toxoid was given 7 days after the injection of diphtheria prophylactic. Less interference occurred in another group primarily stimulated with T.A.B. vaccine.

A secondary stimulus of diphtheria prophylactic suppressed the response to two injections of tetanus toxoid when the first of these was given at times ranging from 3 to 28 days.

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# EXPERIMENTAL OESOPHAGITIS IN RATS.

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In a recent study of non-specific oesophagitis at autopsy (Lodge, 1955) it was found that the lesion occurred much more frequently than is generally recognised. It appeared that conditions which led to excessive contact between gastric contents and the oesophageal epithelium predisposed to the development of oesophagitis. The present paper reports experiments which were designed to test and clarify these conclusions.

Previous workers on gastric ulcer (Buchner, Siebert and Molloy, 1928 ; Hoelzel and Da Costa, 1937) noted that ulcers developed in the proventriculi of rats when they were starved. It was suggested that deficiency of some factor in the diet, such as protein, was responsible for the ulceration. These workers had not, however, appreciated that the proventriculus, which is lined by squamous epithelium, and acts as a food reservoir, is essentially a dilated portion of the oesophagus, as was suggested by Kammeraad (1942) in his study of the development of the gastro-intestinal tract of the rat. The rat, therefore, seemed suitable for the investigation of oesophagitis. In addition it was sufficiently economical in feeding and housing to permit adequate numbers to be used in each experiment, but large enough and with a satisfactory resistance to shock to permit fairly intricate surgical procedures. It was known from previous work on adrenal function (Langley, Lodge and Woodcock, unpublished observations) that rats could be maintained in good condition for up to three weeks on a diet of glucose and sodium chloride solutions without solid food. It seemed reasonable, therefore, to use this diet, which was deficient in protein, to produce partial starvation in rats. An initial experiment was therefore designed to study the formation of ulcers on this diet, and to compare the pH of the gastric contents with that of a control group of rats on normal diet.

## EXPERIMENTAL.

### *Experiment* 1.—*Solution Feeding.*

Pure bred Wistar rats of both sexes were used. They were kept in cages with two feeding bottles, one containing a solution of 25 per cent glucose in water, the other 25 per cent glucose solution with 0·5 per cent sodium chloride added. Sixty rats were used in the experiment. In addition, a group of 36 litter mates of the experimental animals were used as controls, and were fed on diet No. 41 of the Associated London Flour Millers Ltd. Of the 60 test animals, 5 were killed after 2 days of solution feeding, and further groups of 5 rats were killed on alternate days until the completion of the experiment on the 24th day. The control rats were killed at similar 2-day intervals. The oesophagus, proventriculus and stomach of each animal were opened and any fluid present carefully transferred to an agglutination tube. The pH of the supernatant fluid was estimated using a B.D.H. capillator apparatus. The mucosa was examined and in all cases blocks of oesophagus, proventriculus and stomach were taken for microscopy.

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The results are shown in Table I. It can be seen that of the 60 rats used, 52 (87 per cent) had ulcers, and that from the 6th day onwards all the rats on solution feeding had ulcers when killed. All 52 had ulceration of the proventriculus and 14 also had ulcers in the oesophagus. The average number of ulcers per rat increased the longer the animals were kept on the diet. No ulcers were present in any of the control rats. The mean pH of the gastric contents of the rats on solution feeding was 1·94 (S.D. 0·291) compared with the much higher pH of 3·64 (S.D. 0·277) in the normal animals.

TABLE I.—*Production of Oesophageal and Proventricular Ulcers in Rats after a Diet of Glucose and Sodium Chloride.*

| Number of days on diet. | Number of rats killed. | Number of rats with ulcers. | Average number of ulcers per rat. |
|---|---|---|---|
| 2 | 5 | 0 | 0 |
| 4 | 5 | 2 | 1·6 |
| 6 | 5 | 5 | 5·8 |
| 8 | 5 | 5 | 7 |
| 10 | 5 | 5 | 9·4 |
| 12 | 5 | 5 | 10 |
| 14 | 5 | 5 | 17 |
| 16 | 5 | 5 | 22 |
| 18 | 5 | 5 | 42·6 |
| 20 | 5 | 5 | 47·2 |
| 22 | 5 | 5 | 37 |
| 24 | 5 | 5 | 42 |
| | 60 | 52 | |

*Description of ulcers.*

*Distribution.*—In some animals ulcers were numerous throughout the proventriculus, but they were most frequently present in its upper part and at its junction with the oesophagus. Ulcers occurring in the oesophagus were of similar appearance to those in the proventriculus; they were limited to the lower third and were most frequent just above the proventriculo-oesophageal junction. Rarely a few ulcers were seen in the glandular epithelium of the stomach.

*Macroscopic appearances.*—In the earliest stage there is loss of the normal glistening, translucent appearance of the mucosa and white oedematous patches 1 to 2 mm. in diameter are found. Later irregular ulcers appear in these patches (Fig. 1). The ulcers have a white

## DESCRIPTION OF PLATES.

FIG. 1.—The proventriculus and oesophagus in the specimen on the left contain well defined ulcers. The specimen is from a rat fed on glucose and salt solutions for 10 days; the control specimen, right, without ulcers, is from a litter mate fed on normal diet. × 1½.

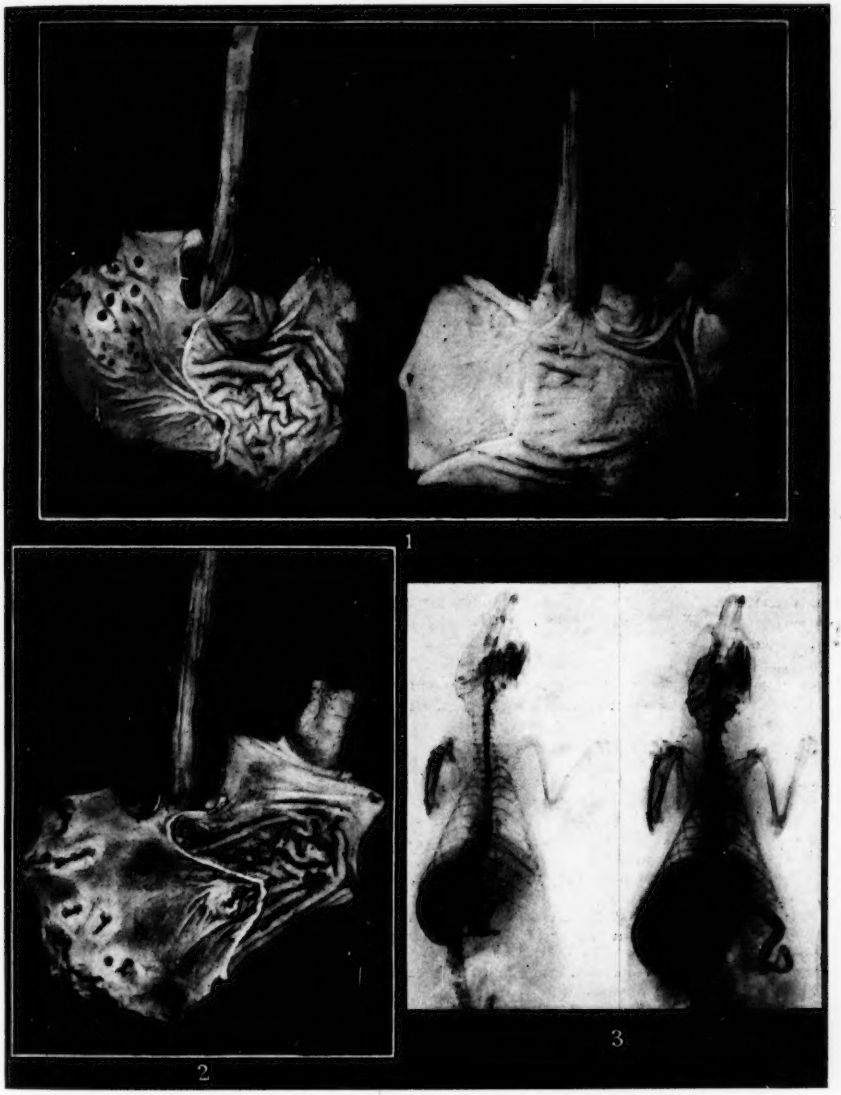
FIG. 2.—The ulcers in the proventriculus are tending to coalesce so that they become oval. In the extreme lower left portion of the specimen this had led to the formation of a large area of ulceration with a ragged margin. The animal was fed on glucose and salt solutions for 16 days. × 1½.

FIG. 3.—There is marked patchy oedema of the lamina propria with thinning and arching of the still intact squamous epithelium. Inflammatory cells infiltrate the lamina propria and extend through the muscularis mucosae into the submucosa. The specimen is from a rat fed on glucose and salt solutions for 4 days. H. and E., × 92.

FIG. 4.—An area of ulceration extends through the muscularis mucosae, with underlying congestion and inflammatory cell infiltration. From an animal fed on glucose and salt solutions for 10 days. H. and E., × 73.

FIG. 5.—Left, dilatation of oesophagus and proventriculus and to a lesser degree of stomach and duodenum after vagotomy with muscle resection. Right, control litter mate showing normal calibre of oesophagus and proventriculus. The X-rays were taken after giving barium sulphate paste through a tube passed into the upper oesophagus.

FIG. 6.—Healing of an oesophageal ulcer by a thin tapering layer of squamous epithelium extending over granulation tissue. The specimen is from a rat which was given glucose and salt solutions for 10 days, followed by a diet of glucose and salt solutions with added 1 per cent sodium bicarbonate for a further 6 days. H. and E., × 95.



Lodge.

Lodge.

or yellow floor and a firm raised margin. There may be congested blood vessels seen as pin point pink spots round the periphery of the lesion. The lesions tend to coalesce and produce elongated ulcerated areas measuring up to 5 mm. in diameter (Fig. 2).

*Microscopic appearances.*—The earliest change is a patchy thickening of the lamina propria due to oedema. The overlying epithelium is thinned and bulges to produce the raised white areas noted macroscopically (Fig. 3). There is diffuse infiltration of the lamina propria by polymorphonuclear leucocytes and lymphocytes, similar to that seen in the early stages of oesophagitis in man. As the lesion progresses the inflammatory cells become localised, and the overlying epithelium breaks away to form an ulcer, with a floor composed of granulation tissue (Fig. 4). As the ulcers increase in size the inflammatory cells infiltrate more widely and may extend deeply into the muscularis externa.

The lesion of the rat oesophagus produced by this diet was associated with lowering of the pH of the gastric contents and possibly resulted from it. To test this suggestion a further experiment was devised to attempt to prevent ulceration by raising the pH of the stomach contents.

*Experiment 2.—Solution Feeding with the Addition of Sodium Bicarbonate.*

The rats were fed on similar solutions to those used in the previous experiment, but with the addition of 1 per cent sodium bicarbonate to each solution. Groups of 5 rats were killed at 2-day intervals as previously. The results are shown in Table II. Of 60 rats only 8 (13·3 per cent) developed any ulcers, all of which were in the proventriculus, and the average number of ulcers per rat was much smaller than in the previous experiment. The mean pH of the gastric contents of these animals was raised to 5·94 (S.D. 1·9). This is a higher pH than that of 3·64 found in the group of normal rats and confirms the supposition that the occurrence of the ulcers was determined by the acidity of the gastric juice in contact with the proventricular and oesophageal epithelium.

TABLE II.—*Production of Oesophageal and Proventricular Ulcers in Rats after a Diet of Glucose, Sodium Chloride and Sodium Bicarbonate.*

| Number of days on diet. | Number of rats killed. | Number of rats with ulcers. | Average number of ulcers per rat. |
|---|---|---|---|
| 2 | 5 | 0 | 0 |
| 4 | 5 | 0 | 0 |
| 6 | 5 | 0 | 0 |
| 8 | 5 | 0 | 0 |
| 10 | 5 | 0 | 0 |
| 12 | 5 | 0 | 0 |
| 14 | 5 | 0 | 0 |
| 16 | 5 | 3 | 0·8 |
| 18 | 5 | 0 | 0 |
| 20 | 5 | 0 | 0 |
| 22 | 5 | 3 | 2·6 |
| 24 | 5 | 2 | 2·4 |
| | 60 | 8 | |

The more frequent formation of ulcers in the proventriculus than in the oesophagus in Exp. 1 further supports the theory that the ulcers were due to the effects of gastric juice, for the proventriculo-oesophageal junction is narrow, whereas the junction between proventriculus and stomach is wide, and thus gastric juice can more easily regurgitate into the proventriculus than into the oesophagus. It appeared that an increased flow of gastric contents back through the proventriculus into the oesophagus should increase the extent of oesophageal ulceration. A special operation was devised for this purpose, as it was found that vagotomy alone did not produce sufficient dilatation of the proventriculo-oesophageal junction.

*Experiment 3.—Vagotomy with Muscle Resection.*

The rats were anaesthetised with intraperitoneal Nembutal (pentobarbitone sodium) and ether by inhalation. Using an aseptic technique the oesophagus and proventriculus were

exposed through a mid-line incision. Bilateral vagotomy was performed. A segment of muscle 1 mm. in width was then resected from the whole circumference of the oesophagus immediately above the proventriculo-oesophageal junction. Care was taken not to incise the mucosa. After operation it was noted that many rats, although taking a normal diet well, continually regurgitated fluid containing particles of food, so that the fur round their mouths and necks became matted and discoloured. X-ray examinations were made post-operatively after giving barium sulphate paste by stomach tube. They showed very marked dilatation of the proventriculus and the oesophagus throughout its length (Fig. 5). The oesophagus also appeared elongated, indicating a relaxation of all the muscle elements, and in some animals there was dilatation of the stomach and duodenum.

The rats were killed at intervals of 2 days to 12 months after the operation. At autopsy there was gross dilatation of the oesophagus and proventriculus and in some animals the oesophagus was filled with food and fluid to the level of the pharynx. No ulceration or inflammatory cell infiltration was seen in any of the specimens on macroscopic or microscopic examination.

It was appreciated that the cutting of the vagi involved in the operation would interfere with the secretion of acid by the stomach but it was decided to study the effects of combining the operative procedure with solution feeding.

*Experiment* 4.—*Vagotomy with Muscle Resection and Solution Feeding.*

The glucose and sodium chloride solutions with added bicarbonate were given to 36 rats for 2 days to empty the proventriculus and stomach of food residue. The operation was then performed as in the previous experiment, and the glucose and salt solutions without bicarbonate which had been found to produce ulcers were given post-operatively.

Groups of 3 rats were examined at intervals of 2 to 24 days after operation. No ulcers were produced in any of the rats and the average pH of the gastric contents was 3·96 (S.D. 0·397). It was clear that the operation reduced acid secretion and so prevented ulcer formation.

In the next experiment vagotomy and muscle resection to produce regurgitation were combined with the feeding of an acid and pepsin solution. This mixture was given to overcome the rise in pH caused by the vagotomy, and it was hoped that conditions might thus be produced in the oesophagus of the rat similar to those thought responsible for oesophagitis in man.

*Experiment* 5.—*Vagotomy with Muscle Resection and Acid and Pepsin Feeding.*

The operation was performed on 24 rats after 2 days' preoperative feeding on solutions with added bicarbonate as in the previous experiment. Post-operatively the 24 rats were divided into 2 groups. Twelve animals were given glucose and sodium chloride solutions with the addition of a mixture of dilute HCl and glycerine pepsin B.P.C.; the remainder received glucose and salt solutions with dilute HCl but no pepsin. In each case the pH of the solution was adjusted to 1·4. This was found to be the most acid solution which the animals would drink.

Rats were killed in batches of 3 on the 8th, 12th, 16th and 20th days after operation. In the HCl + pepsin group, the numbers of rats with ulcers in each batch were 0, 2, 2 and 3 respectively; in the HCl only group they were 0, 1, 3 and 3. The results were thus similar and they are therefore considered together. Of all the 24 rats 14 (58·5 per cent) were found to have ulcers; 12 had ulcers in the oesophagus and 11 had lesions in the proventriculus. In 2 rats perforation of an ulcer occurred. One of these animals was receiving acid and pepsin, the other had had acid only. In each case the ulcer was in the oesophagus at its lower end, just above the junction with the proventriculus. The average pH of the gastric contents in this experiment was 2·14 (S.D. 0·488). Thus, with reflux of acidified gastric contents the most severe lesions occurred at the lower end of the oesophagus, and HCl without pepsin appeared as effective as the acid pepsin mixture in producing ulceration.

To complete the investigations on the effect of the pH of the gastric juice in the production of these ulcers the effect of the treatment of established ulcers with alkali was studied.

*Experiment* 6.—*Repair of Ulcers by Raising the pH of the Gastric Contents.*

Forty rats were given the glucose and sodium chloride solutions for 10 days so that they would develop ulcers as in Exp. 1. The diet of 20 of the animals was then changed to the

glucose and salt solutions with added 1 per cent sodium bicarbonate which had been found to prevent ulceration. The remaining 20 rats continued as controls on their ulcer-producing solutions. On the 2nd, 4th, 6th and 8th days after the change of diet groups of 5 experimental and 5 control animals were killed. It can be seen from Table III that 9 of the rats

TABLE III.—*The Effect of Sodium Bicarbonate on Ulcers Produced in Rats by a Glucose and Salt Diet.*

| Number of days after change to bicarbonate diet. | Number of animals on solutions with bicarbonate showing ulcers (5 killed each day). | Average number of ulcers in rats on bicarbonate. | Number of animals on solutions without bicarbonate showing ulcers (5 killed each day). | Average number of ulcers in rats not on bicarbonate. |
|---|---|---|---|---|
| 2 | 4 | 5·5 | 5 | 9·5 |
| 4 | 4 | 3 | 5 | 14 |
| 6 | 2 | 4 | 5 | 20 |
| 8 | 1 | 2 | 5 | 36 |

fed on the bicarbonate diet had no ulcers, whereas all the rats in the control series showed lesions. Also, in those rats which showed ulceration after bicarbonate treatment the number of ulcers was much smaller than in the control group. The mean pH of the gastric contents in the rats receiving sodium bicarbonate was 6·1 (S.D. 1·49), whereas that of the control group was 2·1 (S.D. 0·35).

Examination of the proventriculus and oesophagus from bicarbonate-treated animals showed some ulcers with a smooth glistening base suggesting possible re-epithelialisation. Microscopically regenerating epithelium was seen growing over the granulation tissue (Fig. 6) and eventually completely bridging the lesion.

## DISCUSSION.

The experimental methods described in this work in the investigation of oesophagitis avoided the artificial technique of perfusion employed by Ferguson, Sanchez-Palomera, Sako, Clatworthy, Toon and Wangensteen (1950), and the mutilation of pyloric ligature as in Selye's (1938) work. Early death of the animals was not caused as in these authors' procedures, and it was possible to kill animals at predetermined intervals so that the stages in the development of the lesion could be studied.

The findings suggest that the inflammatory lesion which occurs in rats fed only on glucose and sodium chloride solutions is due to the resultant lowering of the pH of the gastric juice. Proof is supplied by the absence of ulcers when this fall in pH is prevented by the addition of sodium bicarbonate to the solutions. The operation of vagotomy with muscle resection, designed to cause reflux from the proventriculus into the oesophagus, incidentally provides additional proof of the importance of gastric acid in the production of the lesion, for the vagotomy despite glucose and salt solution feeding prevented any fall in the pH and no ulceration or inflammation occurred. The suggestion of Hoelzel and Da Costa (1932, 1937) that the ulceration of the proventriculus produced by partial starvation of rats is directly due to deficiency of some factor in the diet is thus disproved. If solution feeding were maintained indefinitely deficiency diseases would undoubtedly be a complication, but within the time limits of the present experiment they were not seen.

The experiments also illustrate the importance of reflux of acid stomach contents in producing the lesions. It appears that in the rat two mechanisms

normally limit contact between gastric juice and the mucosa of the proventriculus. First, despite the absence of any true sphincter at the gastro-proventricular junction, peristaltic waves aided by the deflector action of the "limiting ridge"—the mucosal lip at the gastro-proventricular junction—prevent excessive reflux of gastric contents (Lodge, 1954). Further protection is afforded by the bolus of food which the proventriculus normally contains. This mass not only acts as a mechanical barrier to the entry of fluid into the proventriculus, but also tends to absorb fluid and prevent its contact with the proventricular squamous epithelium. In a similar manner peristaltic movement and the constriction at the proventriculo-oesophageal junction provide an even more effective barrier to the flow of gastric juice back into the oesophagus.

After about two days without solids, the bolus of food stored in the proventriculus is exhausted and fluid from the stomach can enter the proventriculus and bathe its walls. The mucosa of the proventriculus is thus unusually exposed to a gastric juice of increased acidity and a chemical inflammation with eventual ulceration results.

The operation of vagotomy combined with the resection of muscle from the lower oesophagus destroys the barrier to regurgitation and a similar inflammation occurs in the oesophagus. A close parallel can be drawn between the mechanism of this experimentally induced reflux and that which from the evidence of previous work (Lodge, 1955) appears to operate in oesophagitis in man. In the human series it appeared that oesophagitis occurred when there was incompetence of the cardio-oesophageal junction, due to such local causes as gastric intubation or general causes such as loss of muscle tone and decubitus, which exposed the squamous epithelium to excessive contact with the gastric juice. The results of repeated vomiting which produces a forcible and frequent entry of stomach contents into the oesophagus were similar.

The experimental evidence thus appears a sound confirmation of the hypothesis that non-specific oesophagitis in man is produced by excessive contact between the acid of gastric juice and the squamous epithelium of the oesophageal mucosa.

## SUMMARY.

The pH of the stomach contents is lowered when rats are fed on glucose and sodium chloride solutions without solid food. Ulceration then occurs in the oesophagus and proventriculus.

Addition of sodium bicarbonate to the solutions prevents the fall in pH and no ulcers form. Similarly, sodium bicarbonate in the diet produces healing in already established ulcers.

An operation devised to facilitate reflux of gastric contents into the rat oesophagus is described.

This operation, combined with the feeding of acidified solutions, produces lesions maximal in the lower oesophagus.

The appearances of oesophagitis in man are thus reproduced in animals, and the importance of excessive contact between acid gastric juice and the oesophageal mucosa in the aetiology of the disease is demonstrated.

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# AGGLUTINATION OF NORMAL ERYTHROCYTES IN MIXTURES OF ANTIBODY AND ANTIGEN, AND HAEMOLYSIS IN THE PRESENCE OF COMPLEMENT.

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DURING studies on various fractions of culture filtrates of the tubercle bacillus in haemagglutination tests we noticed that mixtures of certain of the antigen preparations and anti-tuberculous sera caused the agglutination of sheep erythrocytes, or their haemolysis in the presence of fresh guinea-pig serum. Neither the active antigen nor the antisera alone had any observable effect on red cells. This paper is an account of preliminary investigations on this haemagglutination reaction. Reasons will be given for considering the reaction distinct from the Middlebrook-Dubos test (Middlebrook and Dubos, 1948).

## MATERIALS AND METHODS.

*Erythrocytes.*—Sheep erythrocytes were collected aseptically in 1·2 vol. of Alsever's solution which had been autoclaved at 10 lb. for 15 min. The composition of this solution was as follows: Dextrose 2·05 g., sodium citrate 0·8 g., sodium chloride 0·4 g., distilled water 100 ml., adjusted to pH 6·1 with 10 per cent citric acid solution.

This sheep blood in suspension was kept for at least two days before use and was thereafter used for up to two weeks. At the beginning of each day an aliquot was taken and after the cells had been washed in saline three times, a 2 per cent suspension was made in saline.

*Buffered saline.*—Buffered saline was prepared by adding 1 vol. of saline to 1 vol. of a solution (0·15-M) of $KH_2PO_4$ and $Na_2HPO_4$ mixed in the proper proportions to produce the desired pH (for pH 7·2, 3 vol. $KH_2PO_4$ solution, 7 vol. $Na_2HPO_4$ solution).

*Antigens.*—These were prepared by Dr. E. Sorkin in our laboratory from virulent human type tubercle bacilli. The P.P.D. was obtained by trichloracetic acid precipitation of a heated culture filtrate. Fraction 65 was obtained from an unheated culture filtrate by precipitation with 76 per cent alcohol, after fractions insoluble in lower concentrations had already been removed. Full details of the preparation of these fractions are given elsewhere (Boyden and Sorkin, 1955; Sorkin and Boyden, 1955).

*Rabbit anti-H37Rv serum.*—This was prepared by repeated intravenous injection of killed tubercle bacilli of the strain H37Rv over a period of two months. The bacilli had been killed with 2 per cent phenol, and then extracted with ether and acetone and re-suspended in 0·5 per cent phenol.

*Guinea-pig sera.*—These were obtained as described in the individual experiments.

*Fractionation of sera.*—A crude fractionation of sera was carried out by precipitation of the globulins with 18 per cent $Na_2SO_4$. The serum to be fractionated was placed in a cellophane dialysis bag and dialysed against a large excess (about 200 ×) of 18 per cent $Na_2SO_4$ solution, which was stirred continuously. After 7 hr. the contents of the bag were centrifuged and the supernatant either discarded or dialysed against water and then saline and kept as the "albumin" fraction. The deposit was re-dissolved in re-distilled water and dialysed once more against 18 per cent $Na_2SO_4$, but this time for 16 hr. The new precipitate was also centrifuged down, and then washed once in 18 per cent $Na_2SO_4$. It was then re-dissolved in as small a volume of re-distilled water as possible, and dialysed against cold running tap water for 2 hr., and then against a large excess of saline at 4° for 18 hr., the saline being

changed once in this time. The contents of the bag were then frozen at −17° until needed, but not for a period longer than three weeks.

*Serum dilutions.*—All sera were inactivated by heating at 56° for 30 min. The globulin fractions were not inactivated.

All sera and globulin preparations were absorbed with sheep cells before testing (1 vol. of packed sheep cells to 1 vol. of serum) by contact for 10 min. at room temperature.

Except where otherwise stated, all serum dilutions were made in a solution of 1 per cent normal rabbit serum (hereafter referred to as NRS) in saline.

*Titrations of antigen preparations for sensitisation of red cells in Middlebrook-Dubos test.*—Dilutions of the test material were made in twofold steps in 0·5 ml. quantities of Locke's solution : 0·5 ml. of 2 per cent sheep red cells was then added to each tube and the mixture incubated for 1 hr. at 37°, during which time the tubes were shaken twice. After incubation the cells were washed four times in saline (unless otherwise stated) and re-suspended to 1 per cent. Of each suspension 0·1 ml. was added to 0·3 ml. of a 1/40 dilution of rabbit anti-H37Rv serum. Appropriate controls were included. After shaking, the tubes were left on the bench and the test read the following morning.

*Inhibition by antigen in the Middlebrook-Dubos test.*—Serial dilutions were made in twofold steps in saline of the preparation to be tested (0·2 ml. per tube), and 0·2 ml. of 1/40 rabbit anti-H37Rv serum was added to each tube. The mixture was shaken and left overnight at 4°. The following day 0·1 ml. of 1 per cent sheep erythrocytes, sensitised by incubation with P.P.D. (0·2 mg./ml. 1 hr. 37°, washed 4 ×) was added to each tube. The results were read the next day.

## RESULTS.

The following experiment demonstrates the effect which is the subject of this paper :—

Two rows of twofold dilutions of a rabbit anti-H37Rv serum (No. H.4) were made in 0·1 ml. quantities in 1 per cent NRS, and 0·1 ml. P.P.D. (1 mg./ml.) in buffered saline was added to each tube in the first row, and to a 1 per cent NRS control ; 0·1 ml. buffered saline was added to each tube of the second row. Two rows of dilutions of normal rabbit serum were similarly treated.

Sheep red cell suspension (0·1 ml. of 0·5 per cent) was added to each tube. The tubes were shaken and allowed to stand overnight on the bench. After examination for haemagglutination the following morning, 0·1 ml. of a 50 per cent dilution in saline of fresh guinea-pig serum was added to each of the tubes, which were shaken and incubated for 30 min. at 37°. The results, in terms of haemagglutination and haemolysis, are shown in Table I.

TABLE I.—*Haemagglutination by Mixtures of P.P.D. and Anti-H37Rv Serum, and Haemolysis in the Presence of Complement.*

| | Dilution of serum. | | | | | | | | Added to each tube. |
|---|---|---|---|---|---|---|---|---|---|
| | 10. | 20. | 40. | 80. | 160. | 320. | 640. | 1280. | |
| Normal rabbit serum (haemagglutination) . | − | − | − | − | − | − | − | − | . P.P.D. 1 mg./ml. |
| Normal rabbit serum (haemolysis) . . | − | − | − | − | − | − | − | − | . „ |
| Rabbit anti-H37Rv serum (H.4) (haemagglutination) . . . . | + | ++ | ++ | ++ | + | − | − | − | . „ |
| Rabbit anti-H37Rv serum (H.4) (haemolysis) . | ++ | ++ | ++ | ++ | + | + | − | − | . „ |

A strong agglutination of the cells occurred when they were added to the mixtures of P.P.D. and concentrations of the antiserum up to 1/160. The agglutination was such that the cells came up in large clumps when the tubes were shaken gently. A characteristic pattern was formed by the agglutinated cells

at the bottom of the tubes, similar to that given by rabbit antisera in the Middlebrook-Dubos test. After addition of complement, the agglutinated cells underwent lysis.

Following this initial observation, sera of five other rabbits, which had been vaccinated at the same time as rabbit " H.4 " and in the same way, were similarly treated. Only two of them showed any analogous effect, and with these the agglutination was very feeble and only occurred between the dilutions of 1/40 and 1/160.

Unfortunately, very little of the serum H.4 was available with which to investigate the reaction further. In the hope of obtaining another active serum, six more rabbits were subjected to a similar course of immunisation with phenol-killed H37Rv. The strong haemagglutinating activity shown by serum H.4 failed to appear in the sera of any of these rabbits, although a weak agglutination was given by three of the sera under the conditions of the experiment outlined above (see Table II, part A).

TABLE II.—*Titration of Five Rabbit Anti-tuberculous Sera, and of Globulin Fractions of these Sera.*

| No. of rabbit. | | Fraction. | Dilutions of serum. 10. | 20. | 40. | 80. | 160. | 320. | 640. | 1280. | 2560. | 5120. | C. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A :* | | | | | | | | | | | | | |
| 5573 | Serum | P.P.D. | − | − | − | − | − | − | − | − | − | − | − |
| 5574 | ,, | ,, | − | − | − | + | − | − | − | − | − | − | − |
| 5575 | ,, | ,, | − | − | − | − | − | − | − | − | − | − | − |
| 5576 | ,, | ,, | − | − | − | + | + | + | + | − | − | − | − |
| 5578 | ,, | ,, | − | − | − | − | + | + | + | − | − | − | − |
| *B :* | | | | | | | | | | | | | |
| 5573 | " Globulin " | P.P.D. | − | − | − | − | − | − | − | − | − | − | − |
| 5574 | ,, | ,, | ++ | ++ | ++ | ++ | + | + | − | − | − | − | − |
| 5575 | ,, | ,, | + | ++ | + | − | − | − | − | − | − | − | − |
| 5576 | ,, | ,, | − | ++ | ++ | ++ | + | − | − | − | − | − | − |
| 5578 | ,, | ,, | ++ | ++ | ++ | + | + | − | − | − | − | − | − |
| *C :* | | | | | | | | | | | | | |
| 5573 | Serum | 65 | − | − | − | + | + | + | + | − | − | − | − |
| 5574 | ,, | ,, | − | + | + | ++ | ++ | ++ | ++ | − | − | − | − |
| 5575 | ,, | ,, | − | − | + | + | ++ | + | ± | − | − | − | − |
| 5576 | ,, | ,, | − | + | ++ | ++ | ++ | ++ | ++ | + | − | − | − |
| 5578 | ,, | ,, | − | + | ++ | ++ | ++ | ++ | ++ | + | − | − | − |

The globulin preparations were diluted so that the first tube contained globulin from the same amount of serum as was present in the first tube of the serum dilutions.

All the albumin fractions, and the pre-immunisation sera and the globulin fractions thereof were negative with both P.P.D. and Fr. 65.

*The Effect of High Concentrations of Normal Serum on the Reaction.*

The results of a few miscellaneous experiments carried out with serum H.4 early in the investigation suggested that components of normal serum might have an inhibitory effect on the reaction if present in high concentration. It was thought that if this was so, the inhibitory factor might tend to mask the specific activity of an antiserum, thus possibly accounting for the very feeble agglutination given by some of the sera. With this thought in mind, the following experiment was set up to test the effect of high concentrations of normal serum on the reaction, using two " haemagglutinating doses " of the serum H.4.

Twofold dilutions were made of normal rabbit serum in 0·2 ml. quantities of saline. 0·1 ml. of 1/80 serum 1/4 H.4 (two " haemagglutinating doses ") was added to each tube, and then 0·1 ml. of P.P.D. 1 mg./ml. After 30 min. 0·1 ml. 1 per cent sheep red cells was added to each tube.

The results showed that normal rabbit serum completely inhibited the haemagglutination reaction up to a dilution of 1/80 under the conditions of this experiment. From this it seemed very probable that, in the case of the weakly reacting sera, some normal component of serum was interfering with the reaction.

### *Haemagglutination by Globulin Fractions of Rabbit Antisera.*

In the hope of dissociating the normal serum inhibitor from the specific antibody responsible for the haemagglutination, a crude fractionation of various anti-H37Rv sera was made by the method described. The globulin and albumin fractions thus obtained were tested with P.P.D. by the same technique for haemagglutination. The concentration in the first tube of the dilutions of sera was adjusted to be equivalent to a 1/10 concentration of serum. The results obtained with the globulin fractions are shown in Table II (part B).

The albumin fractions of all the sera were inactive, while all the globulin fractions but one gave the reaction. The prozone-like effect of the whole serum was not given by most of the globulin preparations. Globulin fractions from the pre-immunisation sera were negative. One of the active globulin fractions from anti-H37Rv (No. 5574) was used as a source of antibody for studying various aspects of the mechanism of this haemagglutination in the experiments which follow.

Subsequent experiments have shown that it is often possible to " unmask " the antibodies responsible for this reaction in sera of immunised rabbits negative in the test, by removing the albumin fraction.

The following experiments were made in an endeavour to elucidate the mechanism of the reaction.

### *Absorption by Erythrocytes of the Active Haemagglutinating Principle from P.P.D.*

It seemed probable that the haemagglutination reaction described was associated with an affinity of the antibody-antigen complex for the red cell surface, and it was thought of interest to determine whether either the antigen or the antibody alone could be adsorbed by the cells.

One ml. of a solution of P.P.D., 4 mg./ml., was treated with 1 ml. of packed washed sheep erythrocytes. After 15 min. at room temperature, the mixture was centrifuged and the supernatant fluid kept. Another 1 ml. of a P.P.D. solution was absorbed twice in this way. These two absorbed solutions were then titrated for the haemagglutination together with an unabsorbed portion of P.P.D. solution. Twofold dilutions of each were made in 0·2 ml. quantities of saline and then 0·2 ml. of 1/160 " globulin " (No. 5574) was added to each tube, followed 30 min. later by 0·1 ml. of 2 per cent suspension of sheep red cells.

The results (Table III) show that even a single absorption of P.P.D. with erythrocytes removes almost all the specific activity responsible for this haemagglutination reaction. The antiserum, when similarly adsorbed, did not appre-

ciably drop in titre. This suggests that, for haemagglutination to take place, the antigen must combine both with the cell surface and with the antibody. It is interesting that the affinity of the antigen for the cell surface is such that it is absorbed even after prolonged contact with a relatively high concentration of antibody.

TABLE III.—*Effect of Absorption of P.P.D. with Red Cells on its Ability* (a) *to cause Haemagglutination in the Presence of Anti-H37Rv Serum;* (b) *to Sensitise Red Cells to Agglutination by Anti-H37Rv Serum (Middlebrook-Dubos Method).*

| Pre-treatment of P.P.D. solutions. | Dilutions of P.P.D. solution. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1. | 2. | 4. | 8. | 16. | 32. | 64. | 128. |
| (a): | | | | | | | | |
| Unabsorbed P.P.D. | ++ | ++ | ++ | + | ± | − | − | − |
| Absorbed once | + | − | − | − | − | − | − | − |
| „ twice | − | − | − | − | − | − | − | − |
| (b): | | | | | | | | |
| Unabsorbed P.P.D. | ++ | ++ | ++ | ++ | + | + | ± | − |
| Absorbed once | ++ | ++ | ++ | ++ | + | + | − | − |
| „ twice | ++ | ++ | ++ | ++ | + | ± | − | − |
| „ four times | ++ | ++ | ++ | + | ± | − | − | − |

Because of the possible relationship of this reaction to the Middlebrook-Dubos test, the two absorbed solutions and the control solution were also tested for the specific activity responsible for the latter test. This was done by titrating directly for sensitising activity and for specific antigen inhibition of haemagglutination (see Materials and Methods).

Under the conditions of this experiment, there was no parallelism between the rate of absorption of the materials responsible for these two reactions. Even after two absorptions, at least 50 per cent of the Middlebrook-Dubos activity remained, in contrast to less than 5 per cent of the other activity.

*Removal of the Active Principle from P.P.D.-treated Red Cells by Washing.*

It was found that if red cells which had been treated with P.P.D., centrifuged and separated from the supernatant fluid, were re-suspended in saline, they could be agglutinated by the globulin fraction (No. 5574), or lysed in the presence of complement. This is not surprising after the demonstration that the antigen responsible for the reaction was adsorbed by normal red cells. However, successive washings in saline resulted in progressive loss of agglutinability.

Cells sensitised by incubation of P.P.D. with erythrocytes at 37° for 1 hr. (*i.e.*, the Middlebrook-Dubos technique) did not lose their sensitivity, even after washing ten times after sensitisation. The following experiment demonstrates these effects :

Two suspensions of erythrocytes were treated with P.P.D. (1 mg./ml.), one at 4° for 15 min., the other at 37° for 1 hr. Both were divided into five equal portions, the first of which was centrifuged once and the cells re-suspended in saline. The other portions were washed one, two, four and ten times respectively. After re-suspension to 2 per cent they were added to dilutions of rabbit globulin No. 5574 (see Table IV).

TABLE IV.—(a) *Effect of Washing Red Cells after Treatment with P.P.D. in the Cold.*

| | Dilution of globulin fraction of antiserum. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5. | 10. | 20. | 40. | 80. | 160. | 320. | 640. |
| Cells not washed . . . | ++ | ++ | ++ | ++ | + | + | ± | − |
| „ washed 1 × . . . | + | ++ | ++ | ++ | + | + | − | − |
| „ „ 2 × . . . | − | + | ++ | + | − | − | − | − |
| „ „ 4 × . . . | − | − | − | − | − | − | − | − |
| „ „ 10 × . . . | − | − | − | − | − | − | − | − |

(b) *Effect of Washing Red Cells after Treatment with P.P.D. for 1 hour at 37°.*

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Cells not washed . . . | ++ | ++ | ++ | ++ | ++ | + | + | − |
| „ washed 1 × . . . | ++ | ++ | ++ | ++ | ++ | + | + | − |
| „ „ 2 × . . . | ++ | ++ | ++ | ++ | ++ | + | + | − |
| „ „ 4 × . . . | ++ | ++ | ++ | ++ | ++ | ++ | + | − |
| „ „ 10 × . . . | ++ | ++ | ++ | ++ | ++ | ++ | + | − |

TABLE V.—*Effect of Trypsin on Activity of P.P.D.*

| | 1. | 2. | 4. | 8. | 16. | 32. | 64. | 128. | 256. |
|---|---|---|---|---|---|---|---|---|---|
| P.P.D. + trypsin, 37° 24 hr. . . | − | − | − | − | − | − | − | − | − |
| P.P.D. only, 37° 24 hr. . . . | ++ | ++ | ++ | ++ | ++ | + | + | − | − |
| Trypsin only, 37° 24 hr. . . . | − | − | − | − | − | − | − | − | − |
| P.P.D. + trypsin (inactivated) 37° 24 hr. . . . . | ++ | ++ | ++ | ++ | ++ | + | ± | − | − |

### *Effect of Temperature and Time of Incubation on the Reaction*

Temperature has been shown to affect markedly the rate of sensitisation of red cells in the Middlebrook-Dubos test, the reaction taking place about 10 times more rapidly at 37° than at 20°. At 4° the reaction is negligible (Boyden, 1951).

Mixtures of P.P.D. and globulin (No. 5574) agglutinated cells as rapidly and to the same titre at 4° as at 37°. The agglutination was slightly stronger when the test was carried out in the cold.

### *Effects of Heat and Trypsin on P.P.D.*

*Heat.*—Although the haemagglutinating effect described was found in P.P.D. which had been obtained from a filtrate of a bacterial culture, which had been heated at 110° for 1 hr., it was found that the activity was destroyed by heating the P.P.D. in solution at 1 mg./ml. at 100° for 1 hr. This treatment had little effect on the haemosensitin responsible for the Middlebrook-Dubos test.

*Trypsin.*—Trypsin was dissolved in saline to give a 0·8 per cent solution. The following mixtures and solutions were then made up: (i) 5 ml. P.P.D. solution + 0·1 ml. trypsin solution; (ii) 5 ml. P.P.D. solution; (iii) 5 ml. buffer pH 8·2 + 0·1 ml. trypsin solution; (iv) 5 ml. P.P.D. solution + 0·1 ml. of inactivated trypsin solution (previously heated to 100° for 30 min.). These four mixtures were then incubated at 37° for 24 hr., after which they were neutralised. After neutralisation the concentration corresponded to 7·5 mg. P.P.D./ml.

The solutions before and after this treatment were titrated as previously for activity when mixed with globulin No. 5574. Mixtures (i) and (iii) were inactive; in (ii) and (iv) P.P.D. retained its activity (Table V). It is thus shown that

incubation of P.P.D. with trypsin results in the disappearance of the haemagglutinating effect. The trypsin treatment did not affect the content of specific substance responsible for the Middlebrook-Dubos test, as measured in an antigen inhibition test.

The experiments described in this section suggest that the mechanism of the reaction described in which a mixture of P.P.D. and homologous antibodies agglutinates normal erythrocytes is different from the mechanism of the reaction responsible for the Middlebrook-Dubos test. Furthermore, it appears that the substances responsible for the two reactions are of different specificities. The evidence is consistent with the view that the Middlebrook-Dubos activity is, under the conditions of these experiments, due primarily to a polysaccharide component of tuberculin, while a protein may be responsible for the other haemagglutinating activity. There is apparently no need for the latter substance to confuse the specificity of the Middlebrook-Dubos test, as it can be removed from the cell surface by repeated washing in saline while the polysaccharide is retained.

*Unheated fractions of tuberculin.*—Since most of the work reported above was completed a number of fractions made from unheated filtrates from cultures of tubercle bacilli have been tested for the activity described. One of these fractions (No. 65) has been particularly studied, as it was found that mixtures of it with rabbit antisera, which had previously given negative results with the P.P.D., had haemagglutinating properties. Table II shows the results of titration by this method, using Fraction 65 as antigen, of the same rabbit sera as were tested with P.P.D. Haemolysis followed the addition of complement.

### *Guinea-pig and Human Sera.*

Sera of 20 guinea-pigs taken at intervals following injection subcutaneously of 5 mg. of living B.C.G. were tested using P.P.D. at 1 mg./ml. All were negative. However, five of them gave positive reactions when tested against Fraction 65 (unheated).

Fifty human sera and globulin fractions thereof have been tested in this haemagglutination test, using both P.P.D. and Fraction 65 as antigens. The sera were from healthy individuals (tuberculin-positive and -negative) and from tuberculous patients. None of them gave a positive reaction.

## DISCUSSION.

These experiments show that certain mixtures of antibody and antigen can cause the agglutination of normal sheep erythrocytes, or, in the presence of fresh guinea-pig serum, their haemolysis. Haemagglutination occurred when sera of certain rabbits injected with phenol-killed tubercle bacilli were mixed with P.P.D. from heated culture filtrates. Only a proportion of sera from rabbits vaccinated in this way gave positive results, and in most cases the haemagglutination, when it occurred, was very weak. Crude globulin fractions from these antisera gave much stronger reactions, apparently because of the removal in the albumin fraction of some non-specific inhibitory material. Certain fractions from unheated culture filtrates of the bacillus were later found to be more active than P.P.D., and to give a strong agglutination even with rabbit antisera which had been completely negative when P.P.D. had been used. Furthermore, haemagglutination

occurred when red cells were added to mixtures of such an unheated fraction and sera from guinea-pigs vaccinated with B.C.G. These guinea-pig sera had given negative results with P.P.D.

The reaction appears to be due to an affinity of the antigen for the surface of the red cell, since most of the activity could be removed from a P.P.D. solution by a single absorption with packed cells. It is interesting that the presence of antibody confers haemagglutinating properties on the system without apparently interfering with the adsorption of the antigen by the cells.

The relationship of this phenomenon to the Middlebrook-Dubos test is of some importance, particularly since Middlebrook has made brief reference to a similar effect in a note on that test (Middlebrook, 1952). In referring to the strong activity of a " polysaccharide extract ", he says: " It is of some interest that the purified sensitizing material is capable of sensitizing red cells to agglutination and lysis even after it has combined with antibody This phenomenon occurs only in the higher dilutions of very active antisera ; and normal serum in high concentration interferes with adsorption of the material on to red cells in the presence as well as in the absence of specific antibody." Thus, the reaction which is the subject of this paper is essentially the same in principle as that described by Middlebrook in the foregoing sentences.

However, these studies seem to show that the mechanism of the Middlebrook-Dubos reaction is different from that of the other haemagglutinating effect. In fact the evidence strongly suggests that the active principles in the two reactions are of different chemical nature and of different specificities serologically. Which of the two substances, or groups of substances, Middlebrook was dealing with in the reaction referred to in the quotation from his paper cannot be deduced from the evidence given.

Favour and his colleagues have reported that a proportion of lymphocytes of very tuberculous animals are susceptible *in vitro* to lysis by tuberculin (Favour, 1951). These workers also showed that the lymphocytes from tuberculous individuals gave rise to a soluble substance which, when mixed with normal lymphocytes, resulted in lysis of some of them. In the present experiments erythrocytes were found to be lysed by mixtures of tuberculin and antiserum. The possible relationship of these two phenomena prompted us to determine whether lysis of guinea-pig lymphocytes occurred in mixtures of the antigen (Fraction 65) and guinea-pig sera active in this haemagglutination test. Although some lysis occurred in all tubes, especially in the presence of antigen, the percentage drop in number of cells was the same in normal serum controls as in the antiserum.

Preliminary studies with human sera suggest that, at least in its present form, the new haemagglutination method is unlikely to be of any use in studying antibodies in man against components of the tubercle bacillus.

## SUMMARY.

Mixtures of tuberculo-protein and some rabbit antisera against the tubercle bacillus agglutinate normal sheep cells. Stronger agglutination is given by globulin fractions of antisera than by whole sera, due to the removal in the albumin fraction of a normal serum inhibitor. If fresh guinea-pig serum is added to the system, haemolysis instead of haemagglutination occurs.

Evidence is presented indicating that the mechanism of this haemagglutination reaction is different from that of the Middlebrook-Dubos test, and that the antigens responsible for the two reactions are of different serological specificity.

This work was carried out with the technical assistance of Elisabeth Kindt.

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# A STUDY OF THE ENHANCING EFFECT OF ADJUVANTS ON ANTIBODY FORMATION IN MICE.

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THE possibility of inhibiting carcinogenesis by immunization with carcinogen-protein conjugates is being investigated in this laboratory (Creech, 1952; Creech, Havas and Andre, 1953). In order to delay or prevent carcinogenesis, it is desirable to achieve optimum antibody levels. Mice are good test animals for studies of tumour production by synthetic carcinogens, but they are poor producers of antibodies. Adjuvants which have been shown to promote the formation and maintain the production of antibodies (Freund and McDermott, 1942; Morgan, 1947; Freund, Stern and Pisani, 1947; Kopeloff and Kopeloff, 1947; Halbert, Smolens and Mudd, 1945 *a*, *b*) were therefore used in the present study. Although they are used extensively in immunological investigations, development of immunity in mice has been generally expressed in terms of protection or titres. We have therefore endeavoured to follow the effect of adjuvants on enhancing and maintaining antibody levels in mice by the quantitative precipitin method.

The effectiveness of aluminium hydroxide was compared with that of Freund's adjuvants in enhancing and maintaining antibody production following different courses of immunization. Since the immunizing capacity of the unsubstituted protein parallels that of moderately substituted carcinogen-protein conjugates (Creech *et al.*, 1953) only horse serum albumin was used as the antigen in the following experiments. Although the data so obtained should also be applicable to protein conjugates from horse and bovine serum albumins, this paper provides general information on antibody levels obtained with an unaltered protein.

## MATERIALS AND METHODS.

*Antigens.*—The following preparations, (*a*) horse serum albumin (HSA) in saline, (*b*) HSA adsorbed on aluminium hydroxide (Marrack, personal communication) and (*c*) HSA suspended in adjuvants (Freund and McDermott, 1942) were employed. In (*b*), 40 g. of alum was dissolved in 400 ml. of water and neutralised with 20 per cent $NH_4OH$. The freshly precipitated aluminium hydroxide was washed twice with distilled water, re-suspended in saline containing 1 g. of protein and made up to 150 ml. with saline, giving a final concentration of 2 mg./protein/0·3 ml. In (*c*), each 0·3 ml. of suspension contained 2 mg. protein in 0·075 ml. of saline, 0·2 mg. of dried heat-killed tubercle bacilli in 0·15 ml. liquid paraffin, and 0·075 ml. of Falba.

*Animals.*—C57BL mice of both sexes aged 4–6 months were immunized in balanced groups of 8–16. The mice were bled either 5 days or 4 weeks after the last injection of antigen. The experiments were planned so that all the animals were exsanguinated at 7 months of age.

*Course of Immunization.*—For each experiment the mice were divided into 4 groups: (1) control, (2) injected with 2 mg. of antigen in 0·2 ml. of saline, (3) injected with antigen suspended in Freund's adjuvants, and (4) injected with the antigen adsorbed on aluminium hydroxide. In the first two experiments (Table I), all but the control mice were injected intraperitoneally at weekly intervals for 4 or 8 weeks with preparations containing 2 mg. of

protein and bled 5 days after the last injection. After two or three intraperitoneal injections with the adjuvant-containing preparations, the abdomens of the mice became greatly distended and further injections proved increasingly difficult. Because of this, and because of the high mortality due to peritonitis, the next series of mice was injected intramuscularly (Table II, Exp. III and IV). They were given either a 4 or 8 weeks' course of immunization;

TABLE I.—*Effects of Adjuvants During 4- and 8-week Courses of Intraperitoneal Injections.*

| | Groups. | Number of injections. | Number of survivors ♀. | Number of survivors ♂. | Total protein ppt./ml. serum. mg. ♀. | Total protein ppt./ml. serum. mg. ♂. | Total protein ppt./ml. serum. Average mg. |
|---|---|---|---|---|---|---|---|
| Experiment I (4 weeks' immunization) | Controls | 0 | 4/4 | 4/4 | 0·00 | 0·05 | 0·03 |
| | HSA + saline | 4 | 8/8 | 7/8 | 0·04 | 0·04 | 0·04 |
| | HSA + Freund adjuvants | 4 | 5/8 | 6/8 | 0·31 | 0·32 | 0·31 |
| | HSA + $Al(OH)_3$ | 4 | 7/8 | 2/8 | 0·15 | 0·18 | 0·16 |
| Experiment II (8 weeks' immunization) | Controls | 0 | 4/4 | 4/4 | 0·22 | 0·13 | 0·18 |
| | HSA + saline | 8 | 8/8 | 8/8 | 0·33 | 0·24 | 0·28 |
| | HSA + Freund adjuvants | 8 | 3/8 | 2/8 | 0·82 | 0·72 | 0·77 |
| | HSA + $Al(OH)_3$ | 8 | 3/8 | 3/8 | 0·80 | 0·98 | 0·89 |

All mice were given 2 mg. of protein per injection once a week and were bled 5 days after last injection.

TABLE II.—*Effects of Adjuvants During 4- and 8-Week Courses of Intramuscular Injections.*

| | Groups. | Number of injections. | Exsanguinated after: 5 days. Total protein ppt./ml. (mg.) serum. | Exsanguinated after: 4 weeks. Total protein ppt./ml. (mg.) serum. |
|---|---|---|---|---|
| Experiment III (4 weeks' immunization) | Control | 0 | 0·15 | 0·15 |
| | HSA + saline | 4 | 0·21 | 0·16 |
| | HSA + Freund adjuvants | 4 | 0·37 | 0·37 |
| | HSA + $Al(OH)_3$ | 4 | 0·22 | 0·19 |
| Experiment IV (8 weeks' immunization) | Control | 0 | 0·15 | 0·09 |
| | HSA + saline | 8 | 0·21 | 0·09 |
| | HSA + Freund adjuvants | 8 | 0·36 | 0·28 |
| | HSA + $Al(OH)_3$ | 8 | 0·24 | 0·32 |

Based on sera from 211 mice surviving from total of 224: 8 mice in control groups with 100 per cent survival, 16 mice in all other groups with 93 per cent survival.
All mice were given 2 mg. protein per injection per week.

half of the animals in each group were bled 5 days after the last injection and the other half were bled 4 weeks later (approximately 16 for each group).

*Analytical procedure.*—The blood from the male and female mice of each group was pooled and analysed separately. A 0·5 to 2·0 ml. amount of serum was set up with 400–1000μg. of the homologous antigen per ml. of serum; the precipitates were assayed by the quantitative precipitin method (Heidelberger, Kendall and Soo Hoo, 1933). It was determined from tests on the supernatants that, at this level, the reactions occurred in the equivalence zone or at antigen excess. After 5–7 days in the cold, the precipitates were centrifuged and an additional 400μg. of antigen was added to the supernatants. Any additional precipitate was added to the original precipitate. The antigen-antibody precipitates were analysed for nitrogen by the micro-Kjeldahl method.

## RESULTS AND DISCUSSION.

It is apparent from Tables I and II that, with the use of adjuvants, measurable amounts of antibody-antigen precipitate were obtained from 1–2 ml. of pooled mouse serum following the injection of 8–16 mg. of horse serum albumin. The mouse antisera were weak in comparison with anti-HSA rabbit sera where, without the use of adjuvants, up to 7 mg. of antigen-antibody precipitate was obtained per ml. of serum (Creech *et al.*, 1953).

From Experiments I and II (Table I) it can be seen that, following an *intraperitoneal* course of injections (*a*) 16 mg. of HSA injected over a period of 8 weeks produced appreciable levels of antibody, (*b*) for the 4-week course of immunization, the highest levels were obtained using Freund's adjuvants, while for an 8-week course, aluminium hydroxide produced the highest levels of antibody, and (*c*) there was no consistent difference in antibody response between female and male mice.

In order to rule out any possible contribution of bacterial growth to the high control values, an additional 48 female and 48 male normal C57BL mice were bled, the serum pooled separately and set up with horse serum albumin. To half of the serum (6 aliquots of 2 ml. each), two drops of merthiolate (1/1000) were added. After seven days the precipitates were analysed as previously described. The total amounts of precipitate in the controls varied from 0·06–0·12 mg. of protein per ml. of serum. The aliquot tubes containing the merthiolate did not give lower nitrogen values. It is assumed therefore that the antibody-antigen precipitate obtained with the sera of the control mice is due to the presence of "normal" antibodies, which cross-precipitate with horse serum albumin.

Comparing the effectiveness of adjuvants for intraperitoneal *vs.* intramuscular injections (Exp. I and II, Table I, *vs.* Exp. III and IV, Table II) it can be seen that after 4 weeks both routes produce similar antibody levels, but after 8 weeks higher levels are obtained following intraperitoneal injections. Since this resulted in severe peritonitis, followed by high mortality, the inflammatory reaction might have non-specifically enhanced the antigenicity of HSA and thus produced an additive effect on that of the adjuvant.

In the third and fourth experiments (Table II), all injections were given intramuscularly. Again, no consistent differences were obtained between male and female mice, and thus only average values are given in Table II. Following 4 weeks' immunization, HSA suspended in Freund's adjuvants produced the highest levels of antibody in mice bled either 5 days after the last injection or following a 4 weeks' rest. After 8 weeks of immunization, HSA mixed with tubercle bacillus adjuvant also showed higher values in the groups of mice bled 5 days after the last injection, while HSA with aluminium hydroxide was little better than the saline solution of HSA. Only when the 8 weeks' period of immunization was followed by 4 weeks' rest was aluminium hydroxide found to be as effective as Freund's adjuvant in enhancing the antigenicity of horse serum albumin.

With the use of both aluminium hydroxide and Freund's adjuvants, antibody levels were well maintained during 4 weeks' rest following an 8-week course of immunization (Table II). This is of particular interest because, during the period of action of the carcinogenic agent, it is considered important to maintain satisfactory antibody levels without having to continue injections of the conjugate.

SUMMARY.

Freund's adjuvants and aluminium hydroxide have been compared for their effectiveness in enhancing the antigenicity of horse serum albumin in C57BL mice.

Following different courses of immunization, antibody levels were measured by the quantitative precipitin method. With either Freund's or aluminium hydroxide adjuvants, antibody levels were well maintained following the injection of 16 mg. of horse serum albumin during an 8-week course of immunization. Intraperitoneal injections sometimes elicited higher antibody levels in mice than intramuscular injections, but they also caused a high mortality.

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# THE EFFECT OF SUCKLING ANAEMIA ON THE PIG'S HEART.

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SEVERE anaemia is known to be accompanied by an increased output of blood from the heart (Sharpey-Schafer, 1944; Wood, 1952), an increase in its external dimensions and an increase in its weight (Ellis and Faulkner, 1939; Hunter, 1946; Wood, 1952). This enlargement has been attributed to an increase in the size of the individual cells (Hall, 1948; Herrmann, 1952). The increase in size of the voluntary muscles during post-natal growth has also been found to be due mainly if not entirely to an increase in the size of the fibres (Hammond and Appleton, 1932), but multinucleated voluntary muscle fibres are rather different from the mononuclear cardiac cells, and the two are not necessarily comparable. Piglets reared on concrete are very liable to a severe hypochromic anaemia at 3 to 5 weeks of age (McGowan and Crichton, 1923; 1924; McGowan, 1924). When some studies were being made of this (Venn, McCance and Widdowson, 1947; McCance and Widdowson, 1951) it was impossible not to be arrested by the size of the heart in these animals and by the opportunities which it offered of investigating the nature of the enlargement. Work on the subject has been going on intermittently since 1946 and the essential results and conclusions are embodied in this paper. Since the work began other papers have appeared on the hypertrophy or regeneration of other organs (Mandel, Mandel and Jacob, 1950; Thomson, Heagy, Hutchison and Davidson, 1953; Morrison and Pearce, 1954).

## MATERIALS AND METHODS.

Seven anaemic and seven normal piglets were used for this work. Most of the comparisons have been made on littermates. The policy generally pursued has been to rear the litter in surroundings likely to induce a severe anaemia and to prevent some of the litter from becoming anaemic by the administration of iron salts by mouth or by the injection of "Ferrivenin". When it proved very difficult, even after taking the most elaborate precautions, to make the piglets sufficiently anaemic by dietary means alone, some of them were bled by vein puncture at the age of about 2 weeks. This however was not a sufficiently easy or successful practice to make it worth while, and it was discontinued. Some of the piglets were obtained from an inbred "large white" colony in which anaemia during suckling has become invariable (Pomeroy, 1954, personal communication). These piglets were compared with some Essex and crossbred piglets of similar ages reared on the same ground.

The cardiac outputs were determined by the Fick principle under Nembutal anaesthesia. A cannula was tied into the trachea and a catheter introduced into the right ventricle from the jugular vein and blood withdrawn simultaneously from the right ventricle and the left femoral artery. The oxygen consumption of the animal was determined volumetrically and the amount of oxygen in the samples of blood by the usual Van Slyke procedure.

The physical and chemical methods have been standard ones or have been described in detail by Harrison (1953 *a*, *b*). Sodium and potassium were determined with a Beckman flame photometer, and haemoglobin estimated by Gibson and Harrison's (1945) method.

## RESULTS.

Table I gives the average body weights of the 7 anaemic and 7 normal piglets. The weights of the hearts, their outputs and gross composition are also given.

The largest "anaemic" heart amounted to 1 per cent of the body weight but heavier ones still are not uncommonly encountered in veterinary practice. The chief difficulty in this investigation has been to get the haemoglobin to fall to a sufficiently low level to stimulate the desirable degree of cardiac enlargement. The cardiac outputs were considerably greater in the anaemic animals but the gross composition of the heart was unchanged. The anaemic organs contained no more fat, no more—or less—water per unit weight than the normal ones, although any one of the three might have been expected from the terms flabby (Boyd, 1932; Herrmann, 1952) or fatty (MacCallum, 1936; Hunter, 1946; White, 1951) frequently used by pathologists in describing the heart in anaemia, and from the flabby appearance of these particular hearts. The amount of water was always found to be greater per unit weight of auricular than of ventricular tissue but the difference was unaffected by the anaemia.

TABLE I.—*Average Body Weights, Haemoglobin Levels, Heart Weights, Outputs, and Gross Composition in Seven Pairs of Normal and Anaemic Piglets.*

| | Normal piglets. | Anaemic piglets. |
|---|---|---|
| Body weight, kg. | 7·2 | 6·0 |
| Weight of heart, g. | 38·8 | 48·0 |
| Heart wt. as per cent of body wt. | 0·54 | 0·80 |
| S.D. | 0·10 | 0·17 |
| Haemoglobin, g./100 ml. | 11·2 | 4·0 |
| | (all 10 g. per 100 ml. or above) | (all below 5 g. per 100 ml.) |
| Cardiac output (ml./kg./min.) | 167 | 497 |
| *Composition of heart.* | | |
| Water g./100 g. | 80·6 | 80·7 |
| Protein (N × 6·25) g./100 g. | 15·2 | 15·7 |
| Fat g./100 g. | 2·7 | 2·3 |

Table II gives the average mineral composition of the hearts. Again there were no important differences. The amount of inorganic iron per 100 g. of fresh muscle was rather variable and the natural comment is, not that the anaemic hearts contained less, but that they contained so much. The difference between the means was in fact not statistically significant.

TABLE II.—*Mineral Components of Normal and Anaemic Hearts.*

| | Normal. | Anaemic. |
|---|---|---|
| Na, mg./100 g. | 105 | 110 |
| K, ,, | 261 | 248 |
| Mg, ,, | 18·6 | 18·8 |
| P, ,, | 225 | 220 |
| Inorganic Fe, mg./100 g. | 1·4 | 1·1 |

Table III shows the composition of the heart muscle cell, determined as described by Harrison (1953 *a*, *b*). This method depends upon the assumption that the amount of deoxynucleic acid (DNA) in each nucleus is the same, and that it does not change throughout life (Harrison, 1953*a*). Hence by determining the concentrations of DNA and protein in the heart tissue, by assuming that the amount of DNA in each nucleus is 6 $\mu g. \times 10^{-6}$, and by making an allowance for the small amount of protein in the extracellular fluids it is possible to arrive at an estimate of the average size of the heart cell.

TABLE III.—*Composition of the Cell in 3 Normal and 3 Anaemic Hearts using Deoxynucleic Acid as the Standard of Reference.*

| | Normal. | Anaemic. |
|---|---|---|
| Weight of heart as per cent of body wt. | 0·52 | 0·68 |
| Haemoglobin g./100 ml. | 10·2 | 4·7 |
| Mass of diploid cell ($\mu g \times 10^{-4}$) | 31·9 | 33·0 |
| Water ,, | 24·3 | 24·5 |
| Glycogen ,, | 0·12 | 0·18 |
| Protein ,, | 6·3 | 6·7 |
| Neutral lipids ,, | 0·48 | 0·52 |
| Phospholipids ,, | 0·62 | 0·77 |
| Ribonucleic acid ,, | 0·15 | 0·18 |
| Deoxynucleic acid ,, | 0·06 | 0·06 |

Again the comment must be, not that the anaemic cells differed from the normal, but that they were similar. There is perhaps a suggestion that the mass of the cell was fractionally larger in anaemia, with corresponding changes in the protein, ribonucleic acid, *etc.*, associated with unit weight of DNA, but these differences were not significant, and the important conclusion from the chemical evidence must be that, although there had been a considerable increase in the size of the organ, there had been no change in the size or in the composition of the individual cells in the organ. This conclusion might have been vitiated had the anaemic hearts been infiltrated with small cells such as lymphocytes or inflammatory leucocytes with a very high nuclear/cytoplasmic ratio, but this explanation of the findings has been excluded histologically. No collections of inflammatory or small cells were found in the ventricle or auricle of a very anaemic and much enlarged heart.

Dr. W. C. Slater very kindly investigated one anaemic and one normal heart for the protein in the mitochondrial fraction, and several enzyme systems. These included succinic oxidase, cytochrome oxidase, α-ketoglutarate oxidase and oxidative phosphorylases. His results showed that there was little difference between them.

## DISCUSSION.

There are several ways in which these hearts might have increased in weight relative to the rest of the body. Firstly the cells might have become larger (*a*) by taking up water and the appropriate mineral salts ; this would be expected to have shown itself by a rise in the percentage of water and a fall in the percentage of protein in the heart muscle but without any change in the total nitrogen/DNA ratio ; (*b*) by adding to the protein and fluids of the cytoplasm in the proportions originally present ; had this happened there would have been no change in the percentage of protein in the organ or in the cell, but a rise in the total nitrogen/DNA ratio. Secondly the cells might have become more numerous without change of size or composition. This would have led to no change in the water-protein-DNA relationships—and this appears to have been what took place. Had the stimulus to hypertrophy been more sudden, or severe, or come at another age or fallen upon another organ (Hammond and Appleton, 1932 ; Campbell and Kosterlitz, 1949–50 ; Mandel *et al.*, 1950 ; Herrmann, 1952 ; Thomson *et al.*, 1953 ; Morrison and Pearce, 1954) the result might well have been different.

There is however another way of looking at the changing proportions of heart weight and body weight brought about by piglet anaemia. The deficiency of

iron might be regarded as delaying the general growth of the body—which it undoubtedly does—rather than stimulating the growth of one organ, *i.e.*, the heart. The apparent hypertrophy might then be regarded as the normal rate of growth taking place in an abnormal environment. This point of view would make the nature of the cardiac hypertrophy—a multiplication of cells—a very reasonable one to have found in piglet anaemia, but it can only be part of the explanation, for the actual weight of the hearts of the anaemic piglets was undoubtedly greater than that of the hearts of their bigger littermate controls.

SUMMARY.

In piglet anaemia the cardiac output increases considerably and the heart grows proportionately faster than the body.

This " hypertrophy " is thought to be due to an increase in the number of normal muscle cells because no change has been found in (*a*) the quantity of water, of protein or of mineral salts in unit weight of heart muscle ; (*b*) the deoxyribonucleic acid—protein—water relationships within the heart.

The authors are indebted to Mr. R. W. Pomeroy for providing some of the young pigs, to Drs. A. B. Morrison and J. Cort for assistance over the cardiac outputs, to Dr. W. C. Slater for determining the oxidative and phosphorylative capacity of the hearts and to Stan and Terry Cowen for their assiduous care of the animals.

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# A CIRCUMSTANCE IN WHICH THE PHENOMENON OF COAGGLUTINATION COULD COMPLICATE THE ANTI-GLOBULIN SENSITISATION TEST BY SIMULATING AGGLUTINATION.

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BORDET and Gengou (1911) described the clumping of red blood cells by mixtures of protein antigens and homologous antisera and gave the name coagglutination to the phenomenon. Neither the protein antigens nor the antisera by themselves clumped the red cells. Bordet and Gengou concluded that the clumping or coagglutination was caused by the "*entraînement*" of the cells by the developing aggregates of the protein antigen-antibody reaction. Once the antigen-antibody complex had precipitated it had no coagglutinating property; this was only present during the *status nascendi* of the precipitate.

In recent work applying the direct and indirect anti-globulin sensitisation test to the detection of pig red cell iso-antibodies some difficulty was encountered in producing a rabbit anti-pig globulin serum which was free of what appeared to be agglutinating action against suspensions of twice washed unsensitised pig red cells. Further study showed that with only once washed red cells the "agglutinating" action was more marked, while with cells washed three times or more the unsensitised cells were quite stable and remained unagglutinated by the rabbit anti-pig globulin serum. This serum had, of course, previously been absorbed free of agglutinins to pig red cells.

This observation suggested to us that the explanation of this difficulty could be found in the coagglutination phenomenon of Bordet and Gengou. Experiments supporting this contention are reported in this paper. Human, ox and horse red cells are not quite so prone to coagglutination as those of the pig and this is presumably the reason that the phenomenon has not, to our knowledge, been reported before by those using the anti-globulin sensitisation test. However, the present study shows that the red cells of most species may be coagglutinated, if less easily than pig cells, and thus the need for adequate washing of the red cells in the execution of the anti-globulin sensitisation test is stressed.

## MATERIALS.

*Red cell suspensions.*

Blood samples were obtained by venepuncture and three parts added to one part acid citrate dextrose solution to prevent clotting. The ox blood was defibrinated. Cell suspensions were prepared either immediately or after the blood had been stored at 4° for up to five days.

*Anti-globulin sera.*

The anti-pig globulin serum was prepared by injecting a rabbit with an alum-precipitated total globulin fraction of pig serum. The anti-human globulin and anti-guinea-pig globulin

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sera were prepared by injecting rabbits with the untreated total serum proteins of man and guinea-pig respectively. For anti-horse globulin and anti-ox globulin sera the rabbits were injected with alum-precipitated total serum proteins. A goat was injected with untreated rabbit serum to produce the goat anti-rabbit globulin serum. All the sera were heat-inactivated at 56° for 30 min. and absorbed free of agglutinins for the cells against which they were tested. These anti-globulin sera were our standard reagents for the detection of incomplete iso-antibodies to the red cells of man and other animal species.

## RESULTS.

*Coagglutination of the red cells of the guinea-pig by mixtures of normal rabbit serum and guinea-pig anti-rabbit serum-proteins serum.*

This system was one of those studied originally by Bordet and Gengou (1911). A guinea-pig anti-rabbit serum-proteins serum which had previously been heat-inactivated was titrated in 0·1 ml. amounts. Five comparable rows or titrations* were set up. To each tube of these titrations 0·1 ml. of a 2 per cent suspension of well washed guinea-pig red cells was added. Finally 0·1 ml. of a 1/2 dilution of heat-inactivated normal rabbit serum, previously absorbed free of antibodies to guinea-pig cells, was added to all the tubes of the first row, a 1/10 dilution to the second row, a 1/100 dilution to the third row, a 1/1000 dilution to the fourth row and a similar volume of saline to all the tubes of the final row.

The contents of all the tubes were mixed and the tubes placed at 37° until the red cells sedimented. The tubes showing clumping or coagglutination of the red cells are shown in Table I. The degree of coagglutination was judged by examination of the sedimented cells with a hand lens ; the readings were confirmed microscopically. It may be seen that there is a definite proportion of soluble antigen to antiserum most favourable to coagglutination. In this experiment the guinea-pig red cells were coagglutinated by the rabbit serum proteins and the homologous antibodies in the guinea-pig serum.

*Coagglutination of suspensions of insufficiently washed unsensitised red cells by anti-globulin sera.*

In the direct or indirect anti-globulin sensitisation test on human red cells (Coombs, Mourant and Race, 1945) a rabbit anti-human globulin serum is added to detect adsorbed human globulin on the washed red cell suspension. If the

TABLE I.—*Coagglutination of the Red Cells of the Guinea-pig by Mixtures of Normal Rabbit Serum and Guinea-pig Anti-rabbit Serum-proteins Serum.*

| | | Reciprocals of dilutions of guinea-pig anti-rabbit serum-proteins serum. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1. | 2. | 4. | 8. | 16. | 32. | 64. | 128. | 256. | Saline. |
| Rabbit serum† | 1/2 | − | ++ | ++ | +++ | +++ | + | w | − | − | − |
| | 1/10 | − | − | w | + | ++ | +++ | +++ | + | w | − |
| | 1/100 | − | − | − | − | − | w | + | +++ | + | − |
| | 1/1000 | − | − | − | − | − | − | − | − | − | − |
| Saline | | − | − | − | − | − | − | − | − | − | − |

Key to Tables I–III. +++ = Very strong clumping. ++ = Strong clumping. + = Moderate clumping. w = Weak clumping. − = No clumping.

† The rabbit serum was heat-inactivated and absorbed free of antibodies to guinea-pig cells. Order of adding reagents as described in text.

* Titration in this paper designates a serial dilution of a serum in constant volume.

cells are in fact not sensitised, and the washing has not been properly carried out, there will be some residual human serum proteins which, together with the red cells and the added rabbit anti-human serum-proteins serum, could form a system theoretically capable of producing coagglutination.

Since the difficulty which we encountered in our attempt to produce a satisfactory rabbit anti-pig globulin reagent for detecting iso-sensitisation of pig red cells suggested this possibility to us, an experiment on this system is reported first.

The rabbit anti-pig globulin serum was prepared as described under *Materials*. It was heat-inactivated and repeatedly absorbed with pig red cells washed 7 times, to remove all antibodies to these cells. The object of the experiment was to set up a series of comparable titrations of the anti-pig globulin serum and to examine the behaviour of each titration against pig red cells which had been washed in saline, with the object of freeing them of pig serum, with varying degrees of thoroughness.

The cell suspensions were prepared from pig blood which had been taken into an acid citrate dextrose mixture and stored at 4° for a few days. A volume of 0·8 ml. of the citrated blood was centrifuged and the plasma discarded. One or two drops of the deposited cells were then added to 1 ml. saline to match a 2 per cent suspension. This constituted Cell Suspension 1. The deposited cells were then washed in 2 ml. saline and after re-centrifuging the supernatant fluid was again discarded. Two or three drops of the deposited cells were added to 1 ml. saline to match the 2 per cent suspension, and this constituted Cell Suspension 2. After a similar wash Cell Suspension 3 was made. A further wash again gave Cell Suspension 4.

Cell Suspension 1 in 0·1 ml. volumes was added to 0·1 ml. volumes of a titration of the rabbit anti-pig globulin serum. Cell Suspensions 2, 3 and 4 were added to similar serum titrations. The contents of the tubes were mixed and the cells allowed to settle at 37°. The tubes in which the cells were clumped are recorded in Table II. Where the cell suspension had not been adequately washed free of pig serum proteins, for instance Suspension 1 and 2, the cells were clumped by the higher concentrations of the anti-pig globulin serum. Even with Suspension 3 the cells were not completely free from clumping. The cells in Suspension 4 sedimented unclumped in all dilutions of the anti-globulin serum. This indicated that the clumping in the other titrations was not due to agglutinins, but due to coagglutination resulting from the presence of small amounts of pig serum proteins in the cell suspensions.

In order to see if this phenomenon was general and could affect the anti-globulin reaction with other cell suspensions, similar experiments were performed with suspensions of the red cells of man, horse, ox, guinea-pig and rabbit, employing the corresponding anti-globulin sera appropriate for detecting iso-antibodies for the particular cells. The results are shown also in Table II.

The tests with guinea-pig and rabbit red cells recorded in Tables II and III were performed in a medium of 3·3 per cent $MgSO_4 7H_2O$ owing to the non-specific clumping of these cells which may occur when the cells are suspended in saline in the absence of serum. It was shown that magnesium sulphate solution did not prevent the coagglutination of human red cells by the appropriate protein antigen-antibody system.

In all the experiments, except that with rabbit cells, the cell suspensions which

TABLE II.—*Coagglutination of Suspensions of Insufficiently Washed Unsensitised Red Cells by Anti-globulin Sera.*

| Anti-globulin sera. | Red cell suspensions. | | Reciprocals of dilutions of anti-globulin sera. 1. | 2. | 4. | 8. | 16. | 32. | 64. | 128. | Diluent. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rabbit anti-pig globulin serum | Pig | Susp. 1 | ++ | ++ | ++ | + | – | – | – | – | – |
| | | 2 | + | + | ++ | ++ | ++ | + | – | – | – |
| | | 3 | – | – | w | w | w | – | – | – | – |
| | | 4 | – | – | – | – | – | – | – | – | – |
| Rabbit anti-human globulin serum | Human | Susp. 1 | ++ | + | w | – | – | – | – | – | – |
| | | 2 | + | ++ | ++ | ++ | + | w | – | – | – |
| | | 3 | – | – | – | – | – | – | – | – | – |
| | | 4 | – | – | – | – | – | – | – | – | – |
| Rabbit anti-horse globulin serum | Horse | Susp. 1 | ++ | ++ | ++ | ++ | – | – | – | – | – |
| | | 2 | – | – | – | – | w | – | – | – | – |
| | | 3 | – | – | – | – | – | – | – | – | – |
| | | 4 | – | – | – | – | – | – | – | – | – |
| Rabbit anti-ox globulin serum | Ox | Susp. 1 | ++ | – | – | – | – | – | – | – | – |
| | | 2 | ++ | ++ | w | – | – | – | – | – | – |
| | | 3 | w | w | w | w | – | – | – | – | – |
| | | 4 | – | – | – | – | – | – | – | – | – |
| Rabbit anti-guinea-pig globulin serum | Guinea-pig | Susp. 1 | ++ | ++ | ++ | ++ | ++ | – | – | – | – |
| | | 2 | – | w | w | w | – | – | – | – | – |
| | | 3 | – | – | – | – | – | – | – | – | – |
| | | 4 | – | – | – | – | – | – | – | – | – |
| Goat anti-rabbit globulin serum | Rabbit | Susp. 1 | – | – | – | – | – | – | – | – | – |
| | | 2 | – | – | – | – | – | – | – | – | – |
| | | 3 | – | – | – | – | – | – | – | – | – |
| | | 4 | – | – | – | – | – | – | – | – | – |

were insufficiently washed, were coagglutinated in the high concentrations of the homologous anti-globulin serum. The degree to which the phenomenon showed itself varied in the different systems. It was most marked with pig cells and was by no means negligible with human cells.

*A measure of the amount of serum which on contamination of the red cell suspension would give rise to coagglutination.*

The following tests were performed in order to obtain some idea of the amount of serum protein remaining in the cell suspensions after washing which would cause the cells to be coagglutinated by the corresponding anti-globulin serum. In the first experiment six titrations of rabbit anti-pig globulin serum in 0·1 ml. volumes were set up. The same volume of a 2 per cent suspension of pig cells washed four times in saline was added to each tube. Finally 0·1 ml. of pig serum diluted 1/100 was added to each tube of the first titration, diluted 1/1000 to the second titration, diluted 1/10,000 to the third and so on, while to the sixth titration 0·1 ml. of saline was added to each tube.

As in the previous experiments the contents of the tubes were well mixed and the tubes placed at 37° until the cells sedimented. The pattern of coagglutination is recorded in Table III. It may be seen that a contamination with as little as 0·1 ml. of a 1/100,000 dilution of pig serum was sufficient to cause marked coagglutination. Such clumping occurring in an anti-globulin sensitisation test where

TABLE III.—*A Measure of the Amount of Serum which on Contamination of the Red Cell Suspension would give rise to Coagglutination.*

| Anti-globulin serum titrated in 0·1 ml. volumes. | 0·1 ml. suspension of well washed red cells. | 0·1 ml. dilutions of normal sera. | Reciprocals of dilutions of anti-globulin sera. 1. | 2. | 4. | 8. | 16. | 32. | 64. | 128. | Diluent. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Rabbit anti-pig globulin | Pig | Pig 1/100 | ++ | ++ | ++ | ++ | + | – | – | – | – |
| | | Pig 1/1,000 | ++ | ++ | ++ | ++ | ++ | + | – | – | – |
| | | Pig 1/10,000 | + | ++ | ++ | ++ | + | w | – | – | – |
| | | Pig 1/100,000 | w | ++ | ++ | ++ | + | – | – | – | – |
| | | Pig 1/1,000,000 | – | – | w | w | – | – | – | – | – |
| | | Saline | – | – | – | – | – | – | – | – | – |
| Rabbit anti-human globulin | Human | Human 1/100 | ++ | + | – | – | – | – | – | – | – |
| | | Human 1/1,000 | ++ | ++ | + | – | – | – | – | – | – |
| | | Human 1/10,000 | +++ | +++ | ++ | ++ | + | – | – | – | – |
| | | Human 1/100,000 | – | w | + | + | + | + | w | – | – |
| | | Human 1/1,000,000 | – | – | – | – | – | – | – | – | – |
| | | Saline | – | – | – | – | – | – | – | – | – |
| Rabbit anti-horse globulin | Horse | Horse 1/100 | +++ | ++ | ++ | ++ | ++ | – | – | – | – |
| | | Horse 1/1,000 | +++ | ++ | ++ | + | – | – | – | – | – |
| | | Horse 1/10,000 | – | ++ | ++ | ++ | ++ | + | – | – | – |
| | | Horse 1/100,000 | – | – | – | – | w | w | – | – | – |
| | | Horse 1/1,000,000 | – | – | – | – | – | – | – | – | – |
| | | Saline | – | – | – | – | – | – | – | – | – |
| Rabbit anti-ox globulin | Ox* | Ox 1/100 | ++ | – | – | – | – | – | – | – | – |
| | | Ox 1/1,000 | ++ | + | – | – | – | – | – | – | – |
| | | Ox 1/10,000 | ++ | ++ | ++ | – | – | – | – | – | – |
| | | Ox 1/100,000 | w | w | + | w | – | – | – | – | – |
| | | Ox 1/1,000,000 | – | – | – | – | – | – | – | – | – |
| | | Saline | – | – | – | – | – | – | – | – | – |
| Rabbit anti-guinea-pig globulin | Guinea-pig | Guinea-pig 1/10 | ++ | + | – | – | – | – | – | – | – |
| | | Guinea-pig 1/100 | ++ | ++ | + | w | – | – | – | – | – |
| | | Guinea-pig 1/1,000 | +++ | +++ | ++ | + | w | – | – | – | – |
| | | Guinea-pig 1/10,000 | – | w | w | w | – | – | – | – | – |
| | | Guinea-pig 1/100,000 | – | – | – | – | – | – | – | – | – |
| | | $MgSO_4$ solution | – | – | – | – | – | – | – | – | – |

* Ox cells of both the agglutinable and inagglutinable type (Coombs, Gleeson-White and Hall, 1951) were coagglutinable to the same degree.

the cells had not been thoroughly washed would be difficult to distinguish from genuine agglutination.

Performing this experiment with the red cells of man and of other animal species and the corresponding anti-globulin sera gave the results also shown in Table III.

In these experiments the order in which the three reagents, anti-globulin serum, red cells and corresponding normal serum, are added is important. Bordet and Gengou (1911) stressed this fact. Added in the order just mentioned the coagglutination is most marked. When the normal serum and the anti-globulin serum are allowed to interact for a short interval before the cells are added the coagglutination is greatly reduced ; if the interval is larger (15 min.) no coagglutination occurs.

## DISCUSSION.

It is perhaps surprising that clumping of red cells due to coagglutination has not been reported previously as a complication to the anti-globulin sensitisation test. There appear to be two reasons for this. First, there seems to be a varied susceptibility to coagglutination on the part of red cells of different animal species, and it was only when we came to work with pig cells that the phenomenon attracted our attention. Secondly, if the cells are well washed before adding the anti-globulin serum (Coombs *et al.* (1945) recommended three washes for human red cells) the opportunity for coagglutination does not arise.

In the light of these experiments it is obvious that the importance of thoroughly washing the cells in the execution of the anti-globulin sensitisation test cannot be over-stressed. Likewise any attempt to shorten the test by reducing the number of washes which the cells receive before addition of the anti-globulin serum must not be undertaken without bearing the possible consequences in mind.

It is most satisfying to us that the academic study of Bordet and Gengou (1911) should find practical significance in relation to the execution of the anti-globulin sensitisation test.

The varied susceptibility of different red cells to coagglutination is of interest and requires further study. Rabbit red cells were not clumped by a system consisting of human sera and rabbit anti-human globulin serum, which was effective in coagglutinating human cells. There are probably also variations in the capability of different precipitating systems to coagglutinate red cells. A study of the intrinsic mechanism of the phenomenon has not been attempted.

Finally, it should be stated that we have used the word coagglutination according to the definition of Bordet and Genou (1911) and not in the same sense as did Wolff-Eisner (1911), Aoki and Konno (1921) and Haurowitz (1950).

## SUMMARY.

Red cells not completely washed free of their surrounding plasma may be clumped by the corresponding anti-globulin serum, itself free of agglutinins to the red cells. The explanation resides in Bordet and Gengou's phenomenon of coagglutination.

The necessity for washing the red cells thoroughly in the execution of the anti-globulin sensitisation test is stressed. The cells of some animal species seem more susceptible to coagglutination than others.

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# THE STIMULATION AND INHIBITION OF THE GROWTH OF *HAEMOPHILUS INFLUENZAE* ON MEDIA CONTAINING BLOOD.*

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Two factors, both present in blood, are required for the growth of *Haemophilus influenzae*. X factor is furnished by the blood pigment, haemoglobin itself being a poor source and haematin a rich one: V factor, shown by Lwoff and Lwoff (1937) to be a codehydrogenase, is also present in the red cells; this substance is also formed by plants and many species of bacteria, and can be extracted from yeast. It is not so widely recognised that blood also contains substances tending to inhibit the growth of *H. influenzae*, despite the original presence of both necessary factors. Terada (1922) showed that horse serum contains an enzyme-like substance which destroys the growth-promoting factor in red cells when this is liberated by lysis. Knorr (1924) confirmed this and showed that the substance destroyed was V factor, that in lemon juice being completely inactivated by serum in 4 hours at 37°. He also showed that the anti-V activity of serum could be removed by heating to 56°, and that the content in the serum of animal species varied, that of sheep being highest, followed in diminishing order by rabbit, guinea-pig, rat, man and pigeon.

An apparently distinct anti-V substance was shown by Meyer (1934) to exist in red cell stroma. This substance, which he assayed by its capacity to neutralise V factor in tomato juice, was unaffected by heating to 56° or 60° but destroyed at $> 70°$. Meyer found that its content in the red cells of man, sheep, ox and pig was high, in those of rabbit and guinea-pig lower, while horse and pigeon cells contained none.

The present work, which developed to involve a study of these anti-V substances, originated in a chance observation during a routine sputum examination. Large colonies of *H. influenzae* were observed in the culture over an area where the horse blood in the medium had undergone lysis. This lysis was eventually traced to the accidental presence of potassium tellurite; some types of glass now used for making Petri dishes strongly adsorb tellurite from a medium containing it, and liberate it when another medium is introduced. When potassium tellurite was deliberately added to horse blood agar in concentrations from 1/400,000 to 1/6,400,000, or when half a plate was surface-spread with two drops of a 1/1200 solution, the growth of *H. influenzae* was greatly enhanced wherever the additions had caused lysis. On the other hand, horse blood which had been lysed some time before addition to the medium scarcely supported growth at all, colonies being barely visible.

* Condensed from the Greenfield Memorial Prize Thesis, 1954, of the Institute of Medical Laboratory Technology.

## MATERIALS AND METHODS.

*Organism.*—A single strain of *H. influenzae*, isolated from the sputum of a child with bronchiectasis, was used throughout.

*Culture media.*—All the experiments described were done using ox heart peptone (Evans) agar, pH 7·4, made by Wright's method as the basic medium, with yeast extract added as stated; 0·25 ml. haemin solution was added to cultures containing diluted cells or serum and to controls. Yeast extract and haemin solution were prepared according to the method described by Everall (1953). Plates were inoculated with a loopful of an approximately 1/500 dilution in saline of an overnight culture in boiled blood broth and spread uniformly with a bent glass rod. The size of the colonies obtained was observed after one day's incubation and assessed according to the symbols explained in Table I. The blood used was horse blood except where otherwise stated.

## RESULTS.

*Effects of lysis by saponin.*

Some experiments were first done, which confirm the conclusions of Terada and Knorr, on the fate of V factor when horse blood is lysed before addition to a medium. Saponin (0·75 ml. 10 per cent solution) was added to 15 ml. blood and the mixture incubated at 37°; at 15-min. intervals up to 1 hr. and again at 1½ and 2 hr. 1 ml. was added to each of two 20-ml. tubes of medium, one of which was then heated to 80° for 5 min. On plates poured from these, colonies were of maximal size up to 45 min., but then diminished to minimal at 2 hr. on unheated medium; on that heated to 80° they were only slightly larger. In a control plate containing whole blood they were minute, but of maximal size when the medium was heated to 80° after adding the blood.

On the other hand, when washed cells were lysed in the same way by saponin, the capacity of the lysate to support maximal growth was not lost; it was, however, removed by adding serum. Conversely, the capacity of whole blood lysed by saponin to support full growth was restored by yeast extract. A summary of these findings is given in Table I.

TABLE I.—*Growth of* H. influenzae *on Media Containing Horse Blood Treated in Different Ways.*

| | Unheated. | Heated to 80°. |
|---|---|---|
| Fresh blood . . . . | + | +++ |
| Lysed „ . . . . | + | + |
| „ „ + yeast extract . | +++ | +++ |
| „ cells . . . . | +++ | +++ |
| „ „ + serum . . | + | + |

In this and other Tables the symbols used denote colony size as follows: — no growth; + minute colonies visible individually only with a hand lens; ++ colonies of intermediate size; +++ colonies ± 2 mm. in diameter.

*Effect of potassium tellurite.*

Plates containing 5 per cent each of whole blood, washed cells, lysed blood and lysed cells (lysis by saponin) were inoculated, and over one half two drops of a 1/1200 solution of potassium tellurite were spread, with results stated in Table II. Tellurite enhances growth only when intact cells are present, and despite the presence of serum; in producing lysis it appears to liberate V factor, which in these conditions retains its activity. This was verified by the following experiment.

TABLE II.—*The Effect of Surface-spread Tellurite on the Growth of* H. influenzae *on Media Containing Horse Blood Treated in Different Ways.*

| | Plain. | With tellurite. |
|---|---|---|
| Whole blood . . . . | + | +++ |
| Washed cells . . . . | + | +++ |
| Lysed blood . . . . | + | + |
| „ cells . . . . | +++ | +++ |
| „ „ + serum . . | + | + |

Two agar plates, one containing 5 per cent of whole blood and the other 5 per cent of previously lysed blood with saponin, were evenly inoculated with *H. influenzae*; one half of each was then surface-spread with two drops of a 1/1200 solution of potassium tellurite and a staphylococcus streaked across both halves. After overnight incubation the plate containing fresh blood showed giant colonies in the vicinity of the staphylococcus and all over the half spread with tellurite; the remaining area showed barely visible growth; V factor is produced by staphylococci and has been liberated from the cells (Fig. 1). On the plate containing lysed blood there were giant colonies only where the staphylococci had provided V factor, tellurite having no effect (Fig. 2). On the other hand, when 0·5 ml. of 1/80,000 potassium tellurite was added to 9·5 ml. blood and incubated at 37° for 2 hr. before adding 1 ml. to agar, there was partial haemolysis but no stimulation of growth. Similarly, when 0·5 ml. 10 per cent saponin was added to ensure complete lysis tellurite did not protect the V factor from the effect of the serum.

The stimulation of growth by tellurite originally seen was thus proved to depend on lysis of red cells with consequent liberation of V factor in the medium. Saponin has the same effect: giant colonies were produced by spreading 2 drops of a 10 per cent solution over one half of a blood agar plate, and formed around a cup containing this solution.

V factor liberated by lysis by either substance retains its activity when the lysis takes place in agar, although activity is rapidly lost when the cells are lysed before addition to the medium. This difference in behaviour required explanation.

*Protection of V factor in agar.*

To determine whether some element in the medium was protective 0·75 ml. blood and 0·1 ml. 10 per cent saponin were added to 5 ml. each of peptone broth, peptone water, saline and distilled water. After 4 hr. at 37° the whole of each

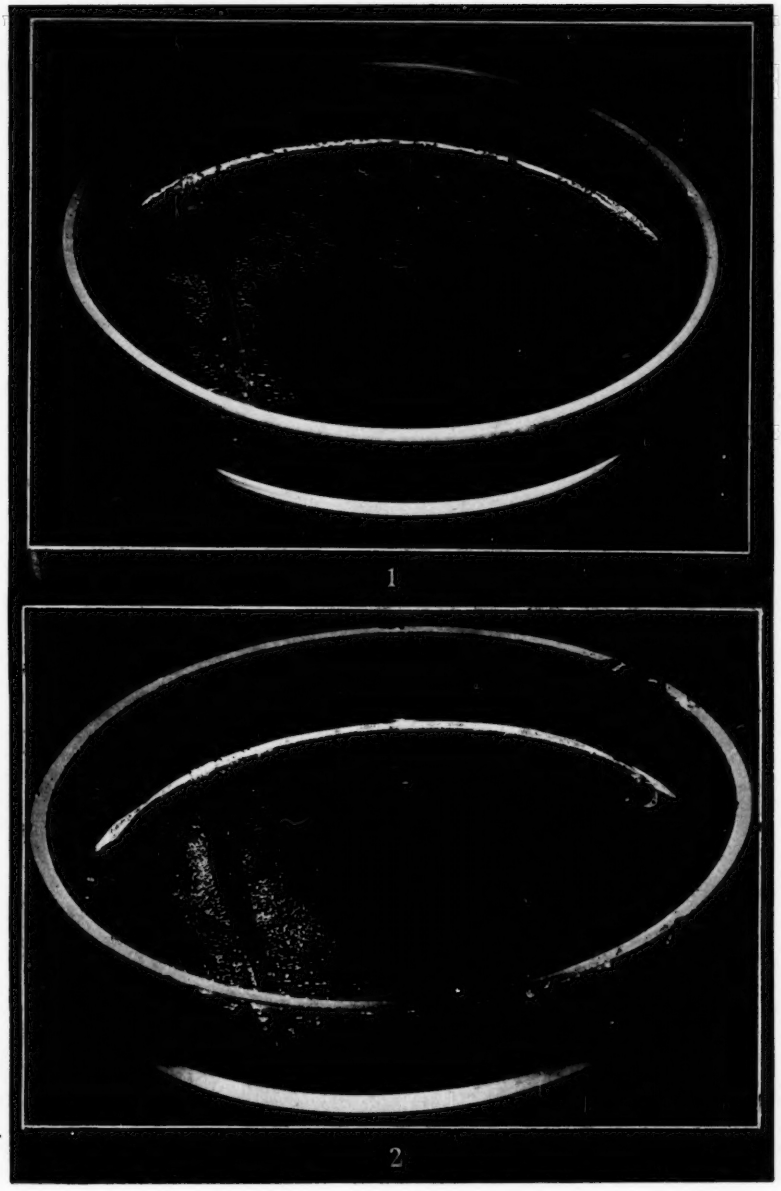
EXPLANATION OF PLATES.

FIG. 1.—*H. influenzae* growing on nutrient agar containing 5 per cent horse blood. The further half of the plate was spread with two drops of a 1/1200 solution of potassium tellurite and a staphylococcus streaked down the left-hand side.

FIG. 2.—*H. influenzae* growing on nutrient agar containing 5 per cent of horse blood previously lysed with saponin. The further half of the plate was spread with two drops of a 1/1200 solution of potassium tellurite and a staphylococcus streaked down the left-hand side.

FIG. 3.—*H. influenzae* growing on nutrient agar containing lysed human cells; the cup contains yeast extract.

FIG. 4.—A cup sensitivity test of *H. influenzae* on nutrient agar containing 5 per cent horse blood; the left-hand side of the plate was spread with two drops of 10 per cent saponin. The cups contain oxytetracycline (50 μg./ml.).



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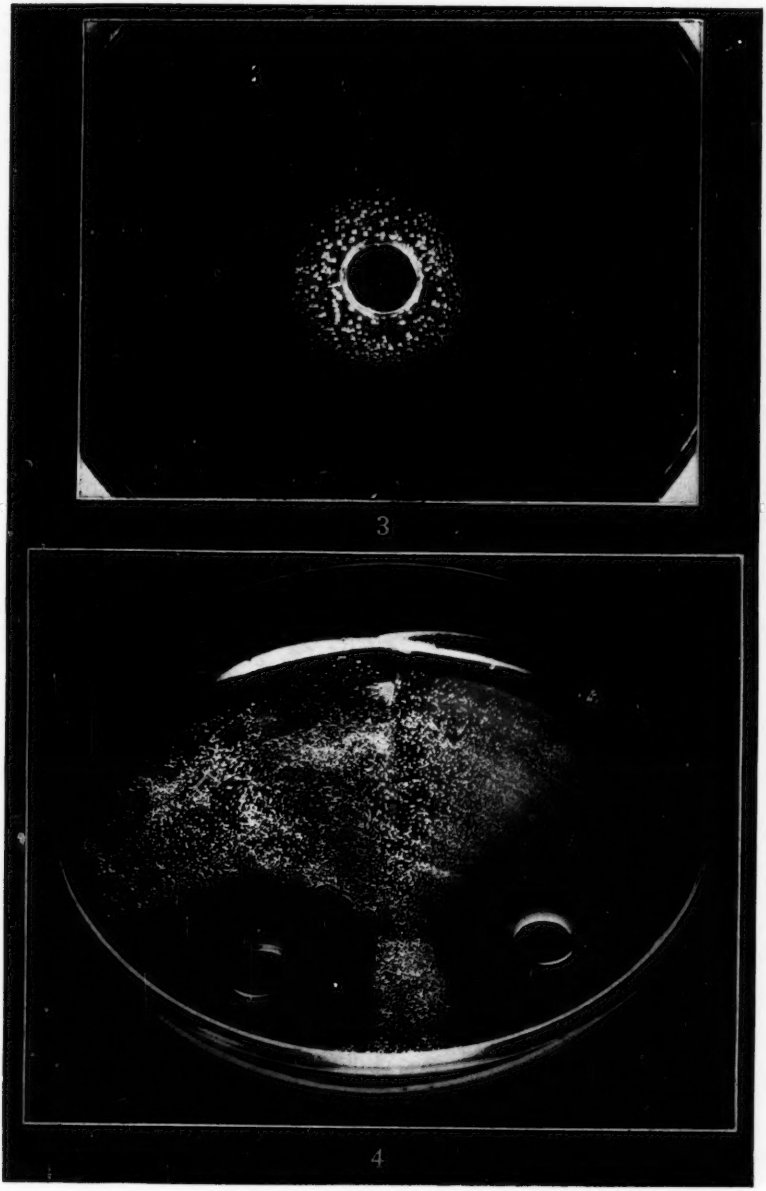

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Waterworth.

mixture was added to 20 ml. agar. Maximal growth was obtained on each medium. This result suggested that dilution might be a factor and accordingly 0·75 ml. blood and 0·1 ml. 10 per cent saponin were added to 0·75, 2·25 and 5·25 ml. distilled water, thereby diluting the blood approximately 1/2, 1/4 and 1/8. These were incubated at 37° for 4 hr. and the whole of each was added to 20 ml. agar. Growth on these plates increased from minute colonies on the control (undiluted blood) and the first dilution to almost full size when the blood was diluted 1/8. This could be reversed: when 2·0 ml. fresh serum were added to 0·75 ml. blood diluted 1/8 the colonies were again minute and if 1·0 ml. was added they were of intermediate size. The results are summarised in Table III.

TABLE III.—*Growth of* H. influenzae *on Media Containing Lysed Horse Blood Showing the Effect of Diluting the Blood on the Inactivation of its V Factor.*

| Dilution of blood. | Undiluted. | 1 in 2. | 1 in 4. | 1 in 8. | 1 in 8 + 1 ml. serum. | 1 in 8 + 2 ml. serum. |
|---|---|---|---|---|---|---|
| Growth | + | + | ++ | +++ | ++ | + |

Similarly, when 7·5 ml. fresh serum were added to agar with 0·5 ml. blood and one half of the plate surface-spread with two drops of 10 per cent saponin, there was some haemolysis but no stimulation of growth; when 2·0 ml. serum were added there was complete lysis but only partial stimulation of growth. That the dilution and not the amount of serum determines the result was proved by the following variation of the experiment in Table III. Washed cells were lysed with saponin, and 1 ml. lysate added to 0·5 ml. serum, 2·5 ml. 1/5 serum and 5 ml. 1/10 serum. Growth on plates containing these mixtures was respectively +, ++ and +++, although the amount of serum was constant.

Thus the power of serum to destroy V factor is lost on dilution, even though the proportion of cells (V factor) to serum remains unchanged. An analogy exists in the lysis of red cells by haemolysin + complement; if the volume of saline added is increased, lysis is delayed or prevented according to the degree of dilution so produced. In blood agar the blood is diluted 1/20 or more, and these experiments show that this is ample to protect V factor from the action of serum.

*Anti-V factor in human blood.*

All the foregoing experiments were done with horse blood. The effect of lysis on human blood was found to be entirely different. Lysis in the medium by surface-spread saponin, instead of producing large colonies as with horse blood, almost or completely inhibited growth. Similarly growth was invisible on medium to which a saponin lysate of washed human cells had been added. That this effect, although evidently due to something in the cells and not the serum, operated by destruction of V factor was suggested by the development of large colonies near contaminants on such plates. The addition of 0·5 ml. yeast extract to 20 ml. agar containing lysed human cells did not restore growth, but a small zone of good growth was obtained around a cup containing yeast extract (Fig. 3) suggesting that either a large amount or a continuous supply of V factor was necessary.

Slopes were then prepared containing 0·15 ml. (3 per cent) of lysed human cells or lysed horse blood devoid of V factor, and different amounts of yeast extract

were added. Whereas (Table IV) 0·1 ml. yeast extract with lysed horse blood gave full growth, 20 times this amount gave only moderate-sized colonies with lysed human cells. It is thus evident that lysed human cells are not only able to destroy a large amount of V factor, but, unlike horse serum, can do this in agar. Human serum, on the other hand, lacks this capacity: 0·5 ml. of each of several samples, incubated at 37° for 4 hr. with 0·5 ml. yeast extract had no effect on the power of the latter to support maximal growth.

TABLE IV.—*Growth of* H. influenzae *on Agar Slopes Containing* 0·15 *ml. Lysed Human Cells or Lysed Horse Blood and Different Amounts of Yeast Extract.*

| | Amount of yeast extract added (ml.). | | | | |
|---|---|---|---|---|---|
| | 0. | 0·1. | 0·5. | 1·0. | 2·0. |
| Lysed human cells . . | − | − | − | + | ++ |
| „ horse blood . . | + | +++ | +++ | +++ | +++ |

Hitherto cells had been lysed by saponin, but in order to control the effect of this and to permit the separation of stroma they were thenceforth lysed by adding 2 vol. distilled water to 1 vol. whole blood or packed cells.

In order to discover what it was in lysed cells which destroyed V factor, stroma and haemoglobin were tested separately: stroma was separated by the addition of 1 per cent acid sodium sulphate to cells lysed with ether and water (Evans, 1952), and washed thoroughly, and haemoglobin by shaking lysed cells with asbestos pulp for 1½ hr., then removing the asbestos by centrifugation. There were barely visible colonies on a plate containing 0·5 ml. stroma and 0·5 ml. yeast extract, but full growth took place when 0·5 ml. haemoglobin and 0·5 ml. yeast extract were added to agar; haemoglobin without yeast extract did not support growth. The anti-V substance is therefore in the stroma.

In view of Terada's findings that the anti-V activity of serum can be removed by heating to 56° for 30 min., it seemed of interest to test the heat resistance of that in human stroma. One ml. freshly lysed cells was added to 20 ml. agar and heated to 56° for 30 min.; there was almost no growth. The experiment was repeated using higher temperatures and including fresh whole blood; horse blood was also tested for comparison. The results (Table V) show that human stroma is not inactivated in 30 min. at 56° or 60°, though 5 min. at 80° suffices. While it is necessary to heat human blood to 80° to make it support full growth, 60° for 15 min. will enable horse blood to do so; haemolysis takes place at 60°, liberating V factor, but if the blood is human this will be destroyed by the stroma unless the latter is first inactivated by greater heat.

TABLE V.—*Growth of* H. influenzae *on Media Containing Human Blood, Human Lysed Cells or Horse Blood, Heated to* 56°, 60° *or* 80°.

| | 56° 30 min. | 60° 15 min. | 60° 30 min. | 80° 5 min. |
|---|---|---|---|---|
| Human lysed cells . . | + | + | + | +++ |
| „ blood . . . | + | + | + | +++ |
| Horse „ . . . | + | +++ | +++ | +++ |

At this point I became aware of the work of Meyer, which confirmed and extended the above findings, and interest then seemed to lie in whether these

two anti-V substances, that in serum and that in some stroma, were in fact the same; beyond saying that it was improbable that the "ferment" was given off from the stroma to the serum, Meyer did not commit himself on this subject. Unlike Terada, Knorr and the writer he found horse serum to have no anti-V activity, but this can be explained by the method he used; this consisted simply of adding 0·5 ml. or less of serum to 5 ml. Levinthal's medium, and it has already been shown that V factor is not inactivated by horse serum when the serum is diluted 1/10 or more.

Meyer found both stroma and serum of the sheep to be highly active; it was therefore decided to investigate sheep's blood to see if it was possible to demonstrate two types of anti-V activity in the blood of one species.

*Anti-V activity of sheep blood.*

Washed sheep cells were lysed with water; both this lysate and the serum inhibited growth when added with yeast extract to agar. When this anti-V activity was titrated, the minimum dilutions of cell lysate and serum respectively which permitted maximal growth after 4 hr. at 37° with 0·5 ml. yeast extract were 1/256 and 1/64, whereas in a control test with horse serum this dilution was, as before, 1/8. This result seems to show that powerful anti-V activity is possessed by both sheep cells and sheep serum, but two properties of the active substance in the serum differentiated it from that in horse serum; it proved to be stable to heat at 56° and 60°, and it acted when diluted in the medium, *i.e.*, without previous admixture with the yeast extract. These facts led to the suspicion that most of the activity of the serum was derived from cells which had undergone lysis before the blood was used.

Red blood cells have the highest proportion of solids of any part of the body, and when they are lysed in distilled water the specific gravity of the solution is so high that the stroma is not deposited by centrifugation without dilution and reduction of pH; it can therefore be assumed that if there is any haemolysis of whole blood the stroma will not be deposited with the cells on centrifuging. The serum used in the foregoing experiments was from defibrinated blood and showed considerable haemolysis; it thus seemed possible that the inhibitory effect observed might in fact be due to the stroma present rather than to the serum itself.

Freshly obtained blood from a single sheep was therefore divided into two portions, one of which was defibrinated and the other allowed to clot; within 3 hr. of bleeding the serum was taken from both and the cells spun down, then half of each was passed through a sintered glass filter (it having been found that Seitz filtration inactivated horse serum). The anti-V activity of these four specimens of serum and the effect on this of heating to 56° for 30 min. was tested as in previous experiments and compared with that of horse serum.

The results of this experiment (Table VI) confirm that if there is no stroma in sheep serum it is no more active than horse serum, and such anti-V activity as it has can be destroyed by heating to 56°. The appearance of the two specimens supported this; the serum from the clotted blood showed no trace of haemolysis, but despite the fact that the serum from the defibrinated blood was separated almost immediately, slight haemolysis was clearly visible by comparison with the other specimen.

TABLE VI.—*Growth of* H. influenzae *on Media Containing* 0·5 *ml. Yeast Extract Previously Incubated for* 4 *hr. at* 37° *with Different Amounts of Sheep Serum before and after Filtration.*

| Serum dilution. | Sheep serum from: Clotted blood. Spun. | Clotted blood. Filtered. | Defibrinated blood. Spun. | Defibrinated blood. Filtered. | Horse serum. Spun. | Horse serum. Filtered. |
|---|---|---|---|---|---|---|
| Undil. | + | + | − | + | + | + |
| 1/2 | ++ | ++ | − | ++ | ++ | ++ |
| 1/4 | +++ | +++ | + | +++ | +++ | +++ |
| 1/8 | +++ | +++ | +++ | +++ | .. | .. |
| 1/16 | +++ | +++ | +++ | +++ | .. | .. |
| Undil. heated to 56° | +++ | +++ | + | +++ | +++ | +++ |

*Ox blood.*

Meyer found both the stroma and serum of ox blood to be active. This blood was tested in the same way, that from a single animal being divided into two portions at the slaughter-house, one being defibrinated. Again, as with sheep blood, serum from the defibrinated portion, which showed some haemolysis, possessed an extra inhibitory effect which was removed by filtration.

An unexpected result was obtained when testing the thermolability of lysed ox cells. Heating for 5 min. at 80° did not affect their capacity to destroy V factor; 0·5 ml. lysed cells and 0·5 ml. yeast extract were then added to agar and heated to 80° for 15 min., and 90° or 100° for 5 min., but none supported growth. Fresh blood was tested in the same way and this also failed to support growth.

*Effects of haemolysis on serum of different species.*

It has already been mentioned that none of several samples of human serum was found to inactivate V factor. In view of these further findings, serum from human defibrinated blood 1 week old and showing considerable haemolysis was tested for anti-V activity and found to have acquired it; moreover this activity was unaffected by heating to 56° for 30 min.

Meyer found serum capable of destroying V factor only in species also possessing highly active stroma, and it is clear that the activity of serum from such animals increases as haemolysis occurs. Sheep cells lyse very easily; defibrinated blood purchased from elsewhere always showed considerable haemolysis on receipt, and it has already been shown that even when separation of the cells is carried out immediately, the process of defibrinating has already caused some haemolysis. The same may well be true of ox blood, and it seems possible that the destruction of V factor by serum observed by Meyer may in fact have been due to stroma. On the other hand, not only do human cells withstand defibrinating without rupturing, but they are also less active than those of either the sheep or ox; consequently a greater degree of haemolysis will be required to produce sufficient stroma to render the serum active. The relative anti-V activity of lysed cells of different species is shown in Table VII.

It is therefore evident that there are two substances in blood which destroy V factor; that in serum is relatively weak and destroyed by heating to 56°, while that in stroma is highly active and unaffected by heating to 56° or 60°. In view of this difference in their thermolability and the fact that it is possible

TABLE VII.—*Growth of* H. influenzae *on Media Containing* 0·5 *ml. Yeast Extract Previously Incubated for* 4 *hr. at* 37° *with Different Amounts of Lysed Cells from Different Species.*

| Dilution of cell lysate. | Human. | Horse. | Sheep. | Ox. |
|---|---|---|---|---|
| Undil. | − | +++ | − | − |
| 1/2 | − | +++ | − | − |
| 1/4 | − | +++ | − | − |
| 1/8 | + | .. | − | − |
| 1/16 | +++ | .. | + | − |
| 1/32 | +++ | .. | + | − |
| 1/64 | +++ | .. | + | + |
| 1/128 | .. | .. | ++ | ++ |
| 1/256 | .. | .. | +++ | ++ |

to have either or both in the blood of any species, it seems improbable that they are the same substance. The anti-V activity of the bloods tested is summarised in Table VIII.

TABLE VIII.—*The Anti-V Activity* (+) *of Lysed Cells and Serum of Different Species.*

| | Human. | Horse. | Sheep. | Ox. |
|---|---|---|---|---|
| Lysed cells | + | − | ++ | ++ |
| Serum | − | + | + | + |

*The choice of a suitable medium for routine use.*

Although *H. influenzae* may grow fairly well on blood agar when mixed with other bacteria, as in cultures from sputum, it will produce at the best only minute colonies when in pure culture and a better medium is necessary. Those in common use include " chocolate " agar, Fildes' extract and Levinthal's medium, all of which require special preparation, and it is suggested that a suitable medium can be produced very simply by surface-spreading half a plate containing horse blood with 2 drops of a 10 per cent solution of saponin. No inhibition of other bacteria has been observed and the method has the advantage that it is possible to identify other organisms present in primary cultures on the side of the plate with no lysis. Fig. 4 illustrates a cup sensitivity test on a culture of *H. influenzae* and shows the difference in growth on the two sides and the clear-cut sensitivity result so obtained. If the medium is required for sensitivity tests using falling dilutions, the saponin can conveniently be added to the agar in bulk, 0·25 ml. of a 10 per cent solution being added to 100 ml. agar with 5 ml. blood, or 0·1 ml. to a 20-ml. tube with 0·5 ml. blood. It is, of course, of great importance that the blood should be from the horse.

The species from which the blood comes is also important when preparing " chocolate " agar. It will be seen from the foregoing findings that ox blood is useless ; human and sheep blood give a satisfactory medium when heated to 80° for 5 min., and horse blood when heated for 15 min. to only 60°, a temperature for which a water bath is available in most laboratories.

*Inhibition of* H. influenzae *by tellurite.*

Fleming (1942) recommended the use of two drops of 1/1000 potassium tellurite spread over half a plate for the inhibition of *H. influenzae.* Although

the tellurite solution used in the experiments described at the beginning of this paper was generally 1/1200, 1/1000 was tried and found to produce slightly fewer but greatly enlarged colonies. The explanation of these conflicting results lies probably in the blood used, and Fleming, in a personal communication, said this was probably human. Tellurite of this strength causes haemolysis and it has been shown above that human blood when lysed not only fails to stimulate the growth of *H. influenzae*, but is actually inhibitory, there being usually no growth at all, although very minute colonies may sometimes appear; it therefore seems probable that the inhibition of this organism observed by Fleming was non-specific.

SUMMARY.

Certain concentrations of potassium tellurite in horse blood agar stimulate the growth of *H. influenzae*; this effect is always associated with haemolysis, and can also be produced with saponin.

If horse blood is lysed before addition to a medium it rapidly loses the capacity to support the growth of *H. influenzae*, owing to inactivation by the serum of V factor liberated from the cells. This inactivation does not occur if the serum is diluted, and dilution explains the persistence of V factor in blood agar lysed by either saponin or tellurite.

Human serum does not inactivate V factor, but a much more potent anti-V substance exists in human red cell stroma. This differs from the substance in horse serum in heat stability and in activity when diluted.

Substances of both types exist in sheep and ox blood.

Slight haemolysis resulting from simple defibrination or storage will liberate enough of the substance in stroma to render the serum (more) inhibitory.

Horse blood agar plates one half of which is surface-spread with saponin are recommended for the primary isolation of *H. influenzae*. When no other organisms have to be identified saponin may be added to agar with the blood; alternatively a medium with horse blood may be heated to 60° for 15 min. The importance of using horse blood is stressed.

I am indebted to Professor L. P. Garrod for facilities for this work, and for advice in the preparation of this paper, and to the Photographic Department for the illustrations.

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# *IN VITRO* STUDIES OF RHEUMATOID SYNOVIUM. PRELIMINARY METABOLIC COMPARISON BETWEEN SYNOVIAL MEMBRANE AND VILLI.

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SOME of the earliest pathological changes which occur in rheumatoid arthritis are proliferative in nature. As the synovial membrane proliferates in response to the disease stimulus more villi are formed and give rise to the florid histological picture. In view of this we felt that the metabolic requirements of villi and membrane would differ quantitatively. We have compared the carbohydrate katabolism of these tissues, since this probably supplies the readily available energy for tissue proliferation.

We have also compared the *in vitro* effects of hydrocortisone (free alcohol) on the two types of tissue. This we felt to be of interest in view of the profound effects of the hormone when administered *in vivo*.

## METHODS.

The tissue was obtained at operation from cases of rheumatoid arthritis undergoing synovectomy or arthrodesis.

Immediately after excision the synovial tissue was placed in ice-cold oxygenated Krebs Ringer phosphate solution (Umbreit, Burris and Stauffer, 1951). As these studies were to be made on a membrane and its villous processes, it was unnecessary to use the classical techniques of tissue slice or mince. A homogenate technique was unsuitable, since the intact cell appears essential for hydrocortisone action (Sutherland and Cori, 1951 ; Miller, 1954). Therefore the chilled tissue was dissected, to remove the membrane from the underlying tissue and to separate the villi if present. The free villi, or membrane strips (5 × 2 mm.), were then washed with fresh ice-cold oxygenated Krebs Ringer phosphate solution. By preparing the tissue as described and storing at 4° in the same oxygenated medium, with the addition of 1 mg./ml. glucose, it usually retained its activity for up to 36 hr. with no significant loss. This was especially true of the villi, whose large surface area allowed free gaseous interchange (Bywaters, 1937).

After re-suspension and thorough mixing, random samples were taken, quickly dried on filter paper, and placed in ice-cold Warburg flasks. The flasks contained 3·5 ml. of oxygenated Krebs Ringer phosphate, with appropriate amounts of substrate and hormone in solution. The hydrocortisone (free alcohol, Merck) was solubilised by the method of Hechter (Miller, 1954). After the addition of the tissue and immediately before incubation, 0·5 ml. of the suspending fluid was removed for zero time estimations on the various substrates, or metabolites, in each flask. The flasks were then attached to the manometers, placed in the bath at 37°, and gassed with 100 per cent oxygen for 10 min. After gassing, a further 10 min. equilibration was allowed. The flasks were then incubated for 1 hr., removed and chilled in an ice bath.

The tissue was separated from the supporting fluid by filtration through glass wool and washed with water. It was then placed in 30 per cent KOH and heated at 100° for 30 min. After solubilisation, aliquots were taken for estimation of total tissue nitrogen by the micro-Kjeldahl method (Hawk, Oser and Summerson, 1952) and glycogen by the method of Walaas and Walaas (1950). The protein in the filtrate was precipitated with zinc and barium, and aliquots taken for the estimation of glucose (Nelson, 1944) and lactic acid, (Barker and Summerson, 1941).

The results of the estimations, including manometric data, were expressed on a tissue nitrogen basis. This gave a more reliable figure than that obtained by the usual method of wet or dry weight reference. The tissue desoxyribonucleic acid content (Schmidt and Tann-

hauser, 1945) was estimated in some cases in an endeavour to assess the variation in cellular number. The results of the total tissue nitrogen paralleled those of the desoxyribonucleic acid values. As the nitrogen estimation was less laborious it was chosen as a standard of reference.

## RESULTS.

The Table shows the rate of $O_2$ uptake (Q $O_2$ (N), *i.e.*, $\mu$l. $O_2$/mg. tissue N/hr.), glucose utilisation and lactic acid production by a number of different tissue samples. Considerable difference in all three values can be seen between the two types of tissue.

TABLE.—*Metabolic Activities of Rheumatoid Villi and Synovial Membrane* in vitro.

| | Villi. | Membrane. |
|---|---|---|
| Q $O_2$ (N) . . . . | 24·7 (25) . | 4·2 (30) |
| $\mu$M. glucose utilised . . . | 1·32 (9) . | 0·52 (10) |
| $\mu$M. lactic acid produced . . | 0·71 (12) . | 0·11 (9) |

Figures are mean values of numbers of observations given in brackets. All are expressed per mg. tissue nitrogen per hr.

In the absence of added substrate during incubation, villous tissue showed a marked decrease in endogenous glycogen. In the membrane the tissue glycogen was too small to allow accurate measurement and no comparison of the rates of utilisation could be made.

The action of hydrocortisone on the $O_2$ uptake of the tissue is shown in Fig. 1. In the presence of glucose the inhibitory effect of the hormone on both types of tissue is diminished.

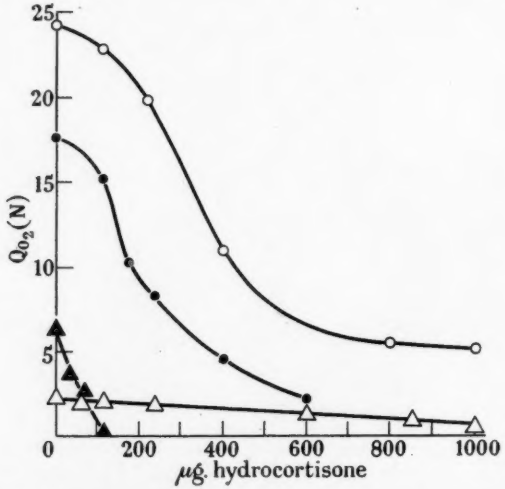


FIG. 1.—The inhibition of oxygen uptake of villi and membrane by hydrocortisone. ○ Villi with glucose substrate. ● Villi with no substrate. △ Membrane with glucose substrate. ▲ Membrane with no substrate.

Fig. 2 and 3 show the inhibition of glucose utilisation and lactate production by varying hormone concentrations.

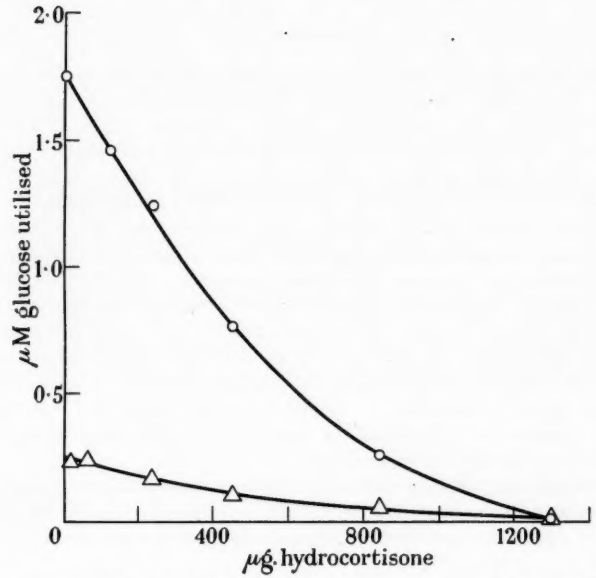


FIG. 2.—Inhibition of glucose utilisation by hydrocortisone. ○ Villi. △ Membrane.

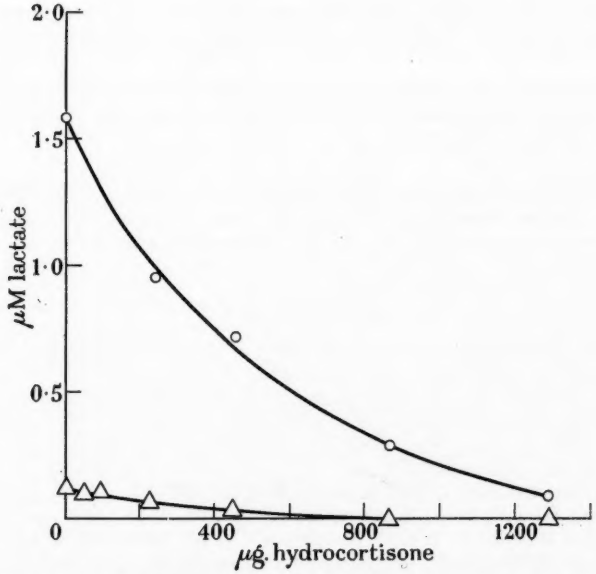


FIG. 3.—Inhibition of lactate production by hydrocortisone. ○ Villi. △ Membrane.

## DISCUSSION.

The results show that villous tissue is more active metabolically than membrane. Thus the former has a higher $O_2$ consumption and utilises more glucose. It is interesting that the patient-to-patient variation in metabolic activity is relatively small when membrane and villous tissue are considered separately. Though these variations are large in comparison with those obtained from homogeneous normal tissues, it is unlikely that such uniformity would be obtained in view of the heterogeneity of the cellular components, and the different phases of the disease process. Experiments performed using a mince of the whole synovium showed wide variations between individual patients. These variations are presumably due mainly to different proportions of membranous and villous tissue.

It is of interest that Miller (1954), using rat thymus lymphocytes, found that hydrocortisone increased aerobic glycolysis and inhibited oxygen uptake. In view of the subsynovial aggregations of lymphocytes in rheumatoid arthritis, similar findings were a possibility. Using the same range of hormone concentrations we have been able to confirm the respiratory inhibition by hydrocortisone, but not an increase in aerobic glycolysis.

Further studies are in progress concerning the action of cortico-steroids on rheumatoid synovium *in vitro*, with special reference to the mechanism of glycolytic inhibition and possible effect on the tricarboxylic acid cycle.

## SUMMARY.

A comparison of the *in vitro* metabolic activity of rheumatoid synovial membrane and villi has been made, with reference to oxygen uptake, glucose utilisation and lactate production. Under these conditions villi showed greater metabolic activity.

The *in vitro* effect of hydrocortisone on these tissues has been demonstrated as it affects carbohydrate katabolism. A similar response to the hormone was given by both types of tissue.

We wish to acknowledge the kind co-operation of the Orthopaedic Consultants of the Bath Clinical area for supplying us with the tissue specimens, and Dr. G. D. Kersley for his constant advice and encouragement.

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# THE FAILURE OF MACROPHAGES TO PRODUCE ANTIBODIES.

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PARTICULATE and many soluble antigens are taken up after injection by cells of the reticulo-endothelial (RE) system (see Roberts, 1954). It is not certainly known whether antibodies are produced by RE cells or, if they are not, what steps intervene between the ingestion of antigens and the production of antibodies by other cells.

## EXPERIMENTAL METHODS.

The present work describes experiments done to see if pure samples of RE cells are able to produce antibodies. Peritoneal macrophages of the rabbit were used as phagocytic cells and formol-killed *Salmonella typhi* as antigen.

*The macrophage.*—The method of Lucké, Strumia, Mudd, McCutcheon and Mudd (1933) as modified by Mackaness (1952) was used to obtain macrophages. An intraperitoneal injection of 50–60 ml. of sterile liquid paraffin was made into young adult rabbits weighing 1·5–2·5 kg.; the exudate so produced was washed out with 300–400 ml. 0·9 per cent NaCl 3–6 days later. The suspension of cells in saline was then filtered through fine gauze and centrifuged; the supernatant was removed and the cells re-suspended in Hanks' solution (Hanks, 1948). The cell suspensions were kept at 4° in screw-capped bottles lined by a thin layer of paraffin wax.

*The antigen.*—A motile strain of *Salm. typhi* was selected by the use of Craigie tubes, and a 24-hr. culture of these organisms in tryptic meat broth was formolised and then heated in a water-bath at 56° for 30 min. The organisms were washed free of formalin and culture medium by centrifuging three times in 0·9 per cent NaCl. The number of organisms in the final suspension was determined by Wellcome opacity tubes, using the figures given by Cunningham and Timothy (1924); this method was found to give results in agreement with those obtained by direct counting of the organisms in a Petrov-Hauser slide. This preparation of *Salm. typhi* H antigen proved to be a satisfactory antigen for the rabbit.

Serum antibody estimations were carried out by the doubling dilution method against a standard agglutinable suspension of *Salm. typhi* H†, equal volumes of diluted serum and this suspension being incubated in a water bath for 2 hr. at 56°. In the earlier experiments, the tubes were then left at 4° overnight, but since this caused no further agglutination, readings were usually taken 30 min. after the tubes had been removed from the water bath.

*Phagocytosis of the antigen.*—The peritoneal exudative macrophages were made to ingest the killed *Salm. typhi* by the following method. The suspended cells were deposited at the bottom of a screw-capped bottle by centrifuging, and re-suspended in a volume of 1 ml. of autologous serum obtained from the rabbit by cardiac puncture before harvesting the exudate. The appropriate number of organisms was then added in a small volume of saline ($< 0{\cdot}5$ ml.) and the mixture centrifuged for 5–7 min. at 1500 r.p.m.; this was found to deposit the cells and all but a few bacteria. The cells and bacteria were left, usually at room temperature, for 30–120 min., at the end of which time extensive phagocytosis of the organisms, as shown in stained smears, had occurred.

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† Obtained from the Standards Laboratory of the Medical Research Council.

*Tissue culture methods.*—The macrophages were maintained in a plasma coagulum contained in small Carrel flasks. Equal volumes (either 0·5 or 1·0 ml.) of cells suspended in Hanks' solution and chilled homologous plasma were mixed and then allowed to clot. The air of the flask was replaced by 5 per cent $CO_2$ in $O_2$ saturated with water vapour. The average thickness of the coagulum, when a total volume of 1 ml. was used, was 1·2 mm. The Carrel flasks were kept at 37° in an incubator constructed so that the cells could be observed, without disturbing the cultures, by means of an inverted microscope.

Sterile precautions were taken throughout these manipulations. In some experiments, crystalline penicillin G and streptomycin hydrochloride, in concentrations of 100 μg./ml., were added to the medium in which the cells were suspended.

## RESULTS.

Between 80 and 95 per cent of the cells in the paraffin-induced peritoneal exudates were macrophages. The term macrophage excludes the non-phagocytic cells of the lymphocytic series and the granulocytes or "microphages". A small proportion of the cells indistinguishable from macrophages when first removed from the peritoneal cavity were, in some exudates, undoubtedly fibroblasts; when cultivated they assumed an elongated form and divided rapidly. It was sometimes difficult to distinguish between the smaller macrophages and the lymphocytes; but only the macrophages, and fibroblasts if present, appeared to survive in culture for any length of time. These macrophages were not seen to divide in these experiments.

### In vitro *experiments.*

When antigen-containing macrophages were cultivated *in vitro* no antibody formation could be demonstrated. Experiments involving the use of exudates from four rabbits were successfully carried out; the following is typical of the series.

Approximately $8 \times 10^7$ macrophages were centrifuged with approximately $1 \times 10^9$ killed *Salm. typhi* and the deposited cells and bacteria left for 60 min. Phagocytosis was nearly complete at the end of this period. The cells were taken up in 4 ml. of 10 per cent serum in Hanks' solution and 1 ml. was placed in each of 4 Carrel flasks with 1 ml. of chilled plasma. The cultures were incubated at 37° and cultures removed at 3, 4, 5 and 6 days. Although there was a growth of fibroblasts, macrophages were still alive and motile at the end of the experiment. The clots were removed and agglutination tests carried out on dilutions of the serum so obtained, but in none of the 4 specimens was *Salm. typhi* H agglutinin demonstrated. No antibody was found in extracts of the cells in the plasma clot when this was ground with 1 ml. of saline and sand. In the other three experiments, the cell : bacteria ratio was approximately 1 : 2, 1 : 10 and 1 : 40 respectively.

That antibodies are not significantly destroyed by macrophages is shown in the following experiment. Normal exudative macrophages were suspended in Hanks' solution containing 10 per cent of a serum with a high titre of H agglutinin. The titre of this mixture of serum and Hanks' solution was 320. The cells were placed in a Carrel flask and an equal volume of normal homologous plasma added. The culture was then incubated for 4 days, the plasma clot freed and serum obtained. The titre of this serum was 80; there had been no significant destruction of antibody.

*Digestion of the organism.*

Antigen-containing macrophages were placed in specially prepared slide chambers according to the method of Mackaness (1952) and incubated at 37°. The cells adhered to the coverslip and preparations were removed and stained at intervals. At 15 min. most of the cells contained bacilli staining well by the May-Grünwald-Giemsa method. At 1 hr. many of the cells contained visible bacteria, but these did not all stain sharply. At 6 hr. most of the bacteria had disappeared, but the outline of faintly-staining bacilli could be seen in some cells; occasional cells still contained well-stained organisms. At 24 hr. no bacteria were to be seen in the majority of cells and at 2 days and after no bacilli or bacterial remnants could be seen. It is apparent that the majority of the peritoneal macrophages can, in these circumstances, digest killed typhoid organisms in less than 6 hr. These cells remained motile for at least 7 days. There was no increased basophilia. Each of the slide chambers contained approximately $6 \times 10^4$ cells and there was a cell : bacteria ratio of approximately 1 : 20. The fluid medium from 6 chambers was pooled at the end of 7 days (1 ml.); this mixed with an equal volume of standard suspension failed to agglutinate the organism.

*Behaviour of macrophages from an immune animal.*

Paraffin-induced exudates were obtained from two animals which had been immunised 2 months previously by an intravenous injection of $10^8$ organisms, their sera agglutinating the organisms at a dilution of 1/320. The cells of the exudate were centrifuged with killed *Salm. typhi*; when phagocytosis had occurred the cells were cultivated in Carrel flasks for 2 and 4 days. No agglutinin could be detected in the medium from any of these cultures.

## In vivo *Experiments.*

*Injection of cells containing antigen into rabbits.*

Exudate macrophages obtained from a normal rabbit were made to ingest $10^9$ killed *Salm. typhi* by the methods described above. These antigen-containing macrophages were suspended in 10 per cent serum in Hanks' solution and injected intraperitoneally into a normal rabbit. The serum-antibody response of this animal is compared in Table I with that of another similarly treated and with that of 3 normal rabbits injected with $10^9$ killed *Salm. typhi* intraperitoneally.

TABLE I.—*Serum Antibody Responses to the Injection of* $10^9$ Salm. typhi.

| No. of rabbit. | Serum agglutinin titres. Days after injection of antigen. 4. | 6. | 10. | 20. |
|---|---|---|---|---|
| 1 | 0 | 2 | 20 | 20 |
| 2 | 0 | 0 | 20 | 20 |
| 3 | 20 | 80 | 160 | 640 |
| 4 | 20 | 80 | 640 | 1280 |
| 5 | 0 | 40 | 320 | 640 |

Rabbits 1 and 2 received an intraperitoneal injection of approximately $5 \times 10^7$ macrophages which had ingested $10^9$ killed *Salm. typhi*. Rabbits 3–5 each received an intraperitoneal injection of $10^9$ killed *Salm. typhi*.

*Injection of antigen into peritoneal exudates of normal rabbits.*

A peritoneal exudate was produced in a normal animal by liquid paraffin; the antigen was then injected into the peritoneal cavity 6 days later. The antibody response was followed for a few days, and the cells harvested and grown in tissue culture for 24 hr.

In a preliminary experiment, samples of the exudate were taken at intervals following the injection of antigen. Some phagocytosis occurred, but the majority of the cells remained free of bacteria. At 24 hr. no bacteria could be seen in the exudate either intra- or extracellularly; there were many granulocytes at this stage. The presence of a paraffin-induced exudate in the peritoneal cavity did not affect the serum antibody response to an injection of $10^{10}$ killed *Salm. typhi*; in 2 rabbits, the serum agglutinin titres were 320 and 2560 when the exudates were removed on the 7th and 9th days respectively. The cells of the exudates were, after washing, cultivated for 24 hr. in Carrel flasks. No antibody could be detected in the culture medium or in the cells extracted by grinding in saline. In a third rabbit the cells were removed on the third day before there was any serum antibody. No agglutinin was formed when these cells were cultivated for 24 hr.

*Macrophages from immune animals.*

In this group of experiments the rabbits were first immunised; later, paraffin was injected into the peritoneal cavity and the exudate removed and assayed for antibody.

*Immunised intravenously.*—In one rabbit, $10^{10}$ organisms were given by this route. A week later, when the serum titre was 640, paraffin was injected intraperitoneally. Four days after this, the serum titre was 1280; the exudate was removed in 600 ml. of saline and the cells, after re-suspending in 2 ml. Hanks' solution, were extracted by grinding with sand. The extracted cells failed to agglutinate the standard suspension.

*Immunised intraperitoneally.*—An exudate was induced in a rabbit 6 days after an intraperitoneal injection of $2 \times 10^9$ organisms; the serum titre at this time was 160. Six days after the injection of paraffin, the exudate was harvested in saline, centrifuged and the cells re-suspended. The cells were then extracted by the Potter homogeniser in 10 ml. of 0·9 per cent NaCl. The titre of the extract was 2. There were originally $6 \times 10^7$ cells present, a wet volume of approximately 0·5 ml. The titre of the cells therefore was probably about 40. The titre of the fluid part of the exudate was estimated to be 14. The serum titre at the time of harvesting the cells was 640. This experiment was repeated in two other rabbits. An intraperitoneal injection of $10^{10}$ organisms was made into one rabbit, and 6 days later, when the serum titre was 1280, 50 ml. paraffin were injected by the same route. On the 10th day after the injection of antigen, the exudate was removed; the serum titre was 1280. The estimated titre of the extracted cells after washing was 40. The second rabbit had been previously immunised with $10^{11}$ organisms given intraperitoneally on two alternate days. Two months later, the same number of organisms was given by the same route and the injection repeated 2 days later. The next day, liquid paraffin was injected intraperitoneally and the exudate harvested at 5 days. The serum titre and the estimated titre of the cells after homogenisation were 5210 and 400 respectively.

This group of experiments shows that the peritoneal exudative cells from animals immunised intraperitoneally do not contain a high titre of antibody. The indication is that they contain less antibody than the serum.

## DISCUSSION.

Peritoneal macrophages which had ingested killed *Salm. typhi in vitro* were kept alive in a solid medium for 3–6 days. No antibody formation could be demonstrated during that period even if the macrophages were taken from an immune rabbit. Beard and Rous (1938), in similar experiments using the Kupffer cell, failed to demonstrate antibody production in tissue culture; their experiments, however, may have been complicated by the use of a self-duplicating antigen, the vaccinia virus. More recently, Fastier (1948) has used various types of rat cells; granulocytes, macrophages, bone-marrow cells and splenic cells. No antibody formation to heat-killed *Salm. paratyphi* B could be demonstrated in fluid culture.

It appears from the present work that antigen-containing exudative macrophages are unable to produce antibody, even when replaced in the tissues of another or of the same animal. It would appear that it is not that the abnormal environment, inevitably found in tissue culture, depresses the cellular activity, as Beard and Rous thought, but that these cells do not, even in favourable conditions, produce significant quantities of antibody.

At the height of antibody formation after intraperitoneal injection of antigen, exudative macrophages neither contain a high titre of antibody, nor are they able to liberate newly formed antibody when kept alive *in vitro*. This is so whether the antigen is injected into an exudate or is injected into the normal peritoneal cavity and the macrophages obtained later.

It is thus probable that the peritoneal exudative macrophage is in the circumstances incapable of antibody formation. This cell must, however, be classified with the RE system, for it readily ingests antigenic and other particulate material and readily segregates neutral red.

In the present experiments the fate of the antigen after its ingestion by this RE cell was followed. The observations provide clear evidence of the ability of these macrophages to digest typhoid bacilli. It was also found that the intracellular position of the antigen lessened its ability to immunise. It is likely that these two phenomena are correlated.

Harris and Ehrich (1946) suggested that the rôle of the RE system is to make particulate antigens soluble and prepare them for the cells actually producing antibody. They base their conclusions on experiments involving the injection of antigen into the foot-pad of the rabbit and the demonstration of soluble antigenic material at the site of injection, in the afferent lymph, and in the popliteal lymph node. However, in other work not reported here, it has been shown that the reaction locally is almost entirely granulocytic at a time when the soluble material was found.

The relationship between cells which segregate antigen, usually RE cells, and those responsible for antibody formation, in many cases the developing plasma cells, still remains unsolved.

## SUMMARY.

Paraffin-induced peritoneal exudates were obtained from rabbits, and the macrophages of these exudates were caused to ingest formalin-killed *Salm. typhi*.

When the antigen-containing macrophages were kept alive in slide chambers, the killed *Salm. typhi* were digested rapidly ; within 6 hr. most of the bacteria had disappeared.

Antigen-containing macrophages were kept alive in tissue culture for periods up to 6 days. No antibody formation could be demonstrated. This was so even when the macrophages were taken from immune rabbits.

Macrophages from rabbits immunised by intraperitoneal injection of killed *Salm. typhi* contained small amounts of antibody.

The intracellular position of the killed organism appeared to reduce its antigenicity.

It was on the suggestion of Professor Sir Howard Florey that this study was undertaken ; I am indebted to him for his advice throughout the work. Dr. G. B. Mackaness very kindly helped in the preparation of slide chamber cultures of macrophages. I have received the able technical assistance of Mr. Peter Harvey.

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# THE PRODUCTION OF INCOMPLETE VIRUS PARTICLES AMONG INFLUENZA STRAINS: CHEMICAL INDUCTION OF THE VON MAGNUS PHENOMENON.

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THE demonstration that multiple infection of cells is not required for the production of incomplete virus (Cairns and Edney, 1952), and that some strains fail to produce non-infective particles even after allantoic inoculation of concentrated seed (Fazekas de St.Groth and Graham, 1953) have rendered untenable that the von Magnus phenomenon was due to auto-interference (von Magnus, 1947, 1951, 1952; Gard, 1953). An alternative interpretation, put forward by Cairns, postulated that the development of infectivity depends on the presence of an unknown factor, initially available in small quantities only and becoming more abundant during continued viral multiplication. This "missing factor hypothesis", accepted in our earlier papers, was vitiated in its turn by the further observation that a set of ten influenza strains, known to yield varying proportions of incomplete virus in the allantois (Fazekas de St.Groth and Graham, 1954*a*), falls into a different gradient when propagated in mouse lungs (Fazekas de St.Groth and Graham, 1955, in preparation). As a consequence, the mechanism underlying incomplete virus production became an open question once again.

One possible approach would be to look for other better known properties of these viruses, hoping that some might follow the same order as the gradients of incomplete virus production. Any correlation, positive or negative, would shed new light on the phenomenon and might thus lead to its eventual understanding. Such systematic studies are under way in our laboratory; the work is however, by nature, a long term project since conclusions cannot be drawn without exploring the possibilities fully.

Another approach relies on the construction of a new working hypothesis, consistent with the newer information. The experimental part here is made up of tests at crucial points, as demanded by theoretical considerations. The present paper reports progress in this direction; it elaborates a hypothesis, shows that it covers known data, and demonstrates that some of its main implications can be verified experimentally.

A preliminary note, summarizing the results, has appeared (Fazekas de St.Groth and Graham, 1954*b*).

## MATERIALS AND METHODS.

Except for a technical change in the performance of haemagglutinin tests, all materials and methods were the same as in the first of our studies (Fazekas de St.Groth and Graham, 1954*a*).

*Haemagglutinin titrations* were performed in transparent plastic trays instead of test tubes. These trays* have depressions of 15 mm. diameter, 11 mm. maximal depth, and perfectly

* Obtained from Prestware Ltd., Lombard St., London. The trays were cast from a die owned by the Medical Research Council of Great Britain.

hemispherical bottoms. Volumes of 0·25 ml. saline were delivered into each depression with an automatic pipette, and doubling dilutions of the test material were set out with the aid of Takátsy's (1950) spiral loop. To each dilution one standard drop (= 0·025 ml.) of a 10 per cent fowl red cell suspension was added from a calibrated dropping pipette, the trays shaken and left standing at room temperature. The pattern of settled cells was read half an hour later.

Apart from making up first dilutions, the human element has been greatly reduced in these titrations and, perhaps for this reason, they show superior reproducibility when compared with our earlier technique. In quality control tests the variance of the method was found to be 0·0123 $\log_2$ units, which gives the error of a single titration as $\pm$ 0·11 $\log_2$ units, *i.e.*, about 8 per cent. As the quantities of the reagents are the same as in the test we used earlier, the two techniques give identical results ; the present method is preferable on account of its increased speed and accuracy.

## THEORETICAL.

The main facts to be considered in connection with incomplete influenza viruses are (*a*) that non-infective virus is produced only in the first cycle of multiplication (Fazekas de St.Groth and Cairns, 1952) ; (*b*) that different strains yield different proportions of incomplete offspring (von Magnus, 1953 ; Fazekas de St.Groth and Graham, 1953, 1954*a*) ; (*c*) that those strains which form incomplete virus will do so only after concentrated inocula (von Magnus, 1947, 1951, 1952, 1953 ; Bernkopf, 1950 ; Cairns and Edney, 1952 ; Fazekas de St.Groth and Graham, 1953, 1954*a*) ; and (*d*) that the proportion of incomplete virus produced depends both on the strain of virus and on the tissue in which multiplication takes place (von Magnus, 1952, 1953 ; Fazekas de St.Groth and Graham, 1954*a*). There are also some negative points, such as the independence of the phenomenon of the type, antigenic relationships and the host range of these viruses (Fazekas de St.Groth and Graham, 1954*a*), as well as of multiple infection of cells (Cairns and Edney, 1952).

Since according to point (*d*) neither virus nor host in itself can be responsible for the production of non-infective particles, the cause must lie in their interaction. Host-virus interaction begins with the adsorption of the infective particle to the surface of an infectible cell. Under the experimental conditions, *i.e.*, in an appropriate ionic medium and far from saturation, this process closely follows the Einstein-Smoluchowski equation and shows, so far as can be determined, 100 per cent collision efficiency over the adsorptive areas of the cell (Fazekas de St.Groth and Stone, unpublished). The situation is thus the same as in the well studied case of bacteriophage and its host-bacterium, or influenza viruses and red cells. As the differences in the rate of adsorption within the group under investigation are so small as to be negligible, it would seem unreasonable to link graded production of incomplete virus later in the infective cycle with differences as slight as these.

While adsorption is practically complete in some minutes, the second stage, viropexis, may last for hours. As evident from the behaviour of the two strains specifically studied (Fazekas de St.Groth, 1948 ; Cairns and Edney, 1952), members of the influenza group exhibit marked differences in this respect. Another observation, seemingly unrelated, is worth noting at this juncture. Cairns (1952) has found that the first cycle of allantoic multiplication takes several hours longer than any of the subsequent cycles. A survey of the literature (Fazekas de St.Groth and Cairns, 1952) revealed that this discrepancy in cycle times was discernible in all published growth curves done in sufficient detail. We have

thus a phase of viral multiplication which both shows strain differences and has unique properties in the first cycle. These differences in viropexis time could then serve as a basis for differences found in the production of incomplete virus particles.

Differences in time, although readily measurable, are of little avail when constructing biological hypotheses, mainly because the rate of these processes depends on more than one factor. Differences in rate, then, have to be translated into something less intangible, and usually this can be done by considering in detail what is happening during the particular process—in this case viropexis. There is good evidence, chiefly from the work of Stone and Ada (1950, 1951, 1952) and Ada and Stone (1950), that once adsorbed the virus particle does not become anchored to the spot it hit first, but continues its Brownian movement in two dimensions, that is, glides along the adsorptive surface. During its random movement the enzymic groupings of the virus come into contact with and act upon substrate groups embedded in the cellular surface. This process—called " browsing " in an earlier paper (Anderson, Burnet, Fazekas de St.Groth, McCrea and Stone, 1948)—goes to completion on red cells and results in spontaneous elution of the virus, since the intact substrate molecules provide the main adsorptive force. Infectible cells, on the other hand, will eventually ingest the virus particle, and it was by analogy to colloidopexis (the uptake by cells of inert submicroscopic particles) that the name viropexis was proposed for the phenomenon (Fazekas de St.Groth, 1948 ; Fazekas de St.Groth and Graham, 1949). Obviously, the amount of substrate split will be directly proportional to the time the virus spends on the outer surface of the cells. Furthermore enzymic destruction will be fairly even over the whole surface, since the virus is less likely to re-visit areas already acted upon while others, with higher adsorptive force, *i.e.*, more substrate, are still available.

Of the last step in this phase, the actual ingestion of the virus, we know little. Phagocytosis is the nearest parallel, and according to this a part of the ectoplasm engulfs the adsorbed foreign particle and folds into the inside of the cell. The only electronmicroscopic study fully supports this view ; Flewett (1953) writes : " The cytoplasm appeared to have flowed completely or partly over the virus particles, and no cell membrane could be clearly distinguished." This occurred $1\frac{1}{2}$ hours after infection with a large dose of virus. It can therefore be maintained in the present state of knowledge that concomitantly with the ingestion of an infective particle a certain area of the cell membrane, too, will find its way into the interior of the cell.

From these considerations, a hypothesis of incomplete virus production might be stated as follows : During viropexis a small area of the ectoplasm folds into the cell, taking with it the infecting particle. If this ingested area contains more than a certain defined minimum of substrate for the viral enzyme, that cell will yield infective particles at the end of the multiplication cycle ; if not, only a fraction of the yield will be infective, the rest consisting of incomplete non-infective particles.

This hypothesis would allow incomplete virus production only in the first cycle, as removal of substrate prior to infection is negligible in later cycles, due to the greatly shortened viropexis time. Since the time spent on the outside of the cell varies from strain to strain, and each strain has a different and characteristic rate of enzyme activity, it follows that the amount of substrate acted

upon will not be uniform, and hence production of incomplete virus can be expected to vary among influenza strains. However, incomplete virus should appear after relatively concentrated inocula only, since the von Magnus phenomenon will not occur unless the average amount of substrate has fallen below a threshold level, as postulated by the hypothesis. Further, since the extent of enzyme action is influenced by the intrinsic properties of both virus and host, as well as by the time available for their interaction, incomplete virus production should depend on both partners. On the other hand, antigenic properties and virulence of these viruses should not bear directly on the phenomenon, which is essentially an aspect and consequence of enzyme-substrate interaction. Neither should multiplicity of infection be a prerequisite since the field of potential enzymic coverage by a single virus particle both extends beyond and is physically independent of cellular territories.

While the new hypothesis is thus found consistent with all known features of the von Magnus phenomenon, its most pretentious tenet, *viz.*, involvement of a chemical grouping of the cell surface in the production of infectivity, requires specific proof. The concluding section of this paper, the experimental part, deals with the design of appropriate tests and gives the findings in detail.

## EXPERIMENTAL.

### *Main experiment.*

The rôle of the receptor substance in endowing newly formed virus with infectivity could be demonstrated in either of two ways :

(1) If the substrate could be shielded from attacks of the viral enzyme without impairing its contribution later in the cycle, cells after such treatment should yield infective virus only, even under conditions where normal cells would produce over 99 per cent incomplete particles.

Or, (2) if the substrate could be inactivated without rendering the cells non-infectible, incomplete particles should issue from infections with strains normally not showing the von Magnus phenomenon and, indeed, even after infections with dilute seed.

As there is no way known at present to block the viral enzyme selectively and not destroy infectivity, the tests had to rely on altering the substrate. The receptor substance for influenza viruses is susceptible to two types of agents. The first group, the " receptor destroying enzymes " of various bacteria (Burnet, McCrea and Stone, 1946 ; Burnet and Stone, 1947 ; McCrea, 1947 ; Stone, 1947), is not particularly suited for the purpose, since destruction of receptors also prevents infection *ipso facto* (Stone, 1948*a*, *b*). For the same reason high concentrations of the second group, the specific carbohydrate reagents, are of no use since they too destroy receptors (Hirst, 1948). Milder treatment with one of the latter, such as the metaperiodate ion, however, results in modification of receptors only ; although the substrate is no longer attacked by the viral enzyme, the adsorptive capacity of the surface remains unchanged (Fazekas de St.Groth, 1949). The main experiment was thus built around the receptor modifying effect of the $IO_4$ ion.

Nevertheless, the outcome of the test could not be predicted directly from the hypothesis, since the effect of $IO_4$ oxidation on the production of infective virus has never been examined. There are two possibilities. Modification of the substrate might not affect the moiety of the receptor substance which later

contributes to the infectivity of newly formed virus. This would realise the first set of conditions. If inactivation were complete, *i.e.*, both aspects of the receptor substance were altered, the second set of conditions would obtain with production of incomplete particles in all circumstances. In either case the outcome should be clear-cut, the expected results differing both from each other and from the normal.

A batch of 11-day eggs was given allantoic inocula of 0·01 mM.-$KIO_4$ (1·0 ml. of 0·01 M-soln.) in saline. The control group received the same volume of saline. Ten minutes later 0·1 ml. 0·3 M glycerol in saline was injected, to neutralise excess periodate. Then sub-groups of six eggs were given large ($10^9$ $ID_{50}$) or small ($10^4$ $ID_{50}$) doses of PR8 (A) or LEE (B) virus. These strains were chosen because they represent the two extremes of the "incomplete virus gradient" in eggs (Fazekas de St.Groth and Graham, 1954*a*), PR8 yielding about 99 per cent non-infective offspring after concentrated inocula, LEE practically none. The eggs were incubated at 35° and harvested at the height of infectivity, *i.e.*, 16 hr. after the large dose and 42 hr. after the small one. Those samples which were not tested for infectivity immediately on harvesting were stored in sealed ampoules on solid $CO_2$. Special care was taken to include representatives from all groups in each set of titrations, since any bias due to possible differences between batches of eggs could thereby be avoided. The results are shown in Table I.

TABLE I.—*Production of Incomplete Virus in Periodate-treated Eggs.*

| Inoculum. | | Untreated eggs (control). | | | Periodate-treated eggs. | | |
|---|---|---|---|---|---|---|---|
| Strain. | Dose ($ID_{50}$). | ID/ml. | HA/ml. | ID/HA. | ID/ml. | HA/ml. | ID/HA. |
| PR8 (A) | $10^9$ | 7·58 | 3·22 | 4·36* | 6·64 | 2·37 | 4·27* |
| | | 7·10 | 3·66 | 3·44* | 6·00 | 2·47 | 3·13* |
| | | 7·29 | 2·86 | 4·43* | 5·83 | 2·67 | 3·16* |
| | | 7·70 | 3·06 | 4·64* | 6·42 | 2·62 | 3·80* |
| | | 6·14 | 2·71 | 3·43* | 6·00 | 2·89 | 3·11* |
| | | 7·33 | 3·24 | 4·09* | 6·10 | 2·44 | 3·66* |
| | | Average ratio: 4·07* ± 0·21 | | | Average ratio: 3·52* ± 0·19 | | |
| „ | $10^4$ | 9·66 | 3·55 | 6·11 | 8·37 | 3·49 | 4·88* |
| | | 8·80 | 3·07 | 5·73 | 7·75 | 3·03 | 4·72* |
| | | 9·70 | 2·68 | 7·02 | 8·25 | 3·75 | 4·50* |
| | | 9·10 | 3·25 | 5·85 | 7·30 | 2·77 | 4·53* |
| | | 8·80 | 3·01 | 5·79 | 7·50 | 2·62 | 4·88* |
| | | 9·13 | 3·34 | 5·79 | 7·75 | 2·62 | 5·13* |
| | | Average ratio: 6·05 ± 0·20 | | | Average ratio: 4·77* ± 0·10 | | |
| LEE (B) | $10^9$ | 8·50 | 2·38 | 6·12 | 7·50 | 3·25 | 4·25* |
| | | 9·13 | 2·95 | 6·18 | 8·17 | 3·10 | 5·07* |
| | | 9·30 | 3·34 | 5·96 | 8·40 | 3·15 | 5·25* |
| | | 9·70 | 2·98 | 6·72 | 8·13 | 3·15 | 4·98* |
| | | 9·50 | 3·04 | 6·46 | 7·89 | 3·22 | 4·67* |
| | | 9·00 | 2·95 | 6·05 | 8·20 | 3·16 | 5·04* |
| | | Average ratio: 6·25 ± 0·12 | | | Average ratio: 4·88* ± 0·15 | | |
| „ | $10^4$ | 9·07 | 3·10 | 5·97 | 7·71 | 3·12 | 4·59* |
| | | 9·25 | 2·97 | 6·28 | 8·10 | 3·13 | 4·97* |
| | | 8·75 | 3·25 | 5·50 | 7·50 | 3·58 | 3·92* |
| | | 9·87 | 3·25 | 6·62 | 8·58 | 3·24 | 5·34* |
| | | 9·08 | 2·77 | 6·31 | 8·17 | 3·22 | 4·95* |
| | | 9·60 | 3·28 | 6·32 | 7·58 | 2·65 | 4·93* |
| | | Average ratio: 6·17 ± 0·16 | | | Average ratio: 4·78* ± 0·20 | | |

Infectivity (ID) and haemagglutinin (HA) titres are in $\log_{10}$ units.

* = Significant production of incomplete virus, *i.e.*, ID/HA $< 5·42$.

The control side of the experiment shows that after small infective doses neither strain produces non-infective particles, the ID/HA ratios being indistinguishable from the theoretical ratio, $10^{6.26}$ (Fazekas de St.Groth and Cairns, 1952). The LEE virus maintains this ratio even after large infective doses, while PR8 forms over 99 per cent incomplete particles in similar circumstances.

In the periodate-treated eggs, on the other hand, all combinations of strains and doses give ID/HA ratios significantly below the normal, that is, the yield contains a great excess of non-infective virus. In terms of the hypothesis this amounts to the realization of the second set of predictions, namely that inactivation of the receptor substance be followed by formation of incomplete virus on infection of the treated tissue.

These results lay particular stress on the host cell's contribution to infectivity. It is clear that the three cardinal features of the von Magnus phenomenon—its occurrence only in the first cycle, only after large inocula, and to different degree with different strains—can be altered by simple chemical treatment of the host, several hours prior to the release of newly formed virus. To establish this statement fully, it has to be shown further (*a*) that the action of the chemical was restricted to the host; (*b*) that cellular receptors have actually been modified under conditions of the experiment; and (*c*) that the virus recovered is the yield of a multiplicative process, and not some remnant of the original seed.

*Subsidiary Experiment I.*

The target of periodate action was tested in the following manner. Batches of six 11-day eggs were given allantoic inocula of 0·01 M-$KIO_4$, or 0·01 M-$KIO_4$ mixed with 0·3 M glycerol, or 0·01 M-$KIO_3$, or saline. Ten minutes later the eggs received $10^9$ or $10^4$ infective doses of PR8 or LEE virus. After incubation at 35° the fluids were harvested, 16 hr. following the concentrated and 42 hr. after the dilute inoculum. Each allantoic fluid was titrated for infectivity and haemagglutinin, with the results shown in Table II.

TABLE II.—*Production of Incomplete Virus after Various Pre-treatments of the Allantois.*

| Inoculum. | | Pre-treatment of allantois. | | | |
|---|---|---|---|---|---|
| Strain. | Dose ($ID_{50}$). | $KIO_4$, 0·01mM. | $KIO_4$, 0·01mM + glycerol, 0·3mM. | $KIO_3$, 0·01mM. | Saline (control). |
| PR8 (A) | $10^4$ | 5·14* | 6·09 | 6·15 | 6·26 |
| LEE (B) | $10^9$ | 4·24* | 6·17 | 6·03 | 6·29 |
| „ | $10^4$ | 4·31* | 6·21 | 6·07 | 6·24 |

The figures show, in $\log_{10}$ units, the average ID/HA ratio of the yield.
* = Significant production of incomplete virus.

Incomplete particles could be detected only after $IO_4$ treatment; all other treatments gave effects indistinguishable from the saline controls.

As a further check, in a second experiment, approximately $10^9$ $ID_{50}$ of the two viruses were treated with 5·0 ml. allantoic fluid containing 1·0 ml. of either 0·01 M-$KIO_4$ or 0·01 M-$KIO_4$ neutralized by glycerol. Ten minutes later 0·1 ml. of 0·3 M glycerol was added to these fluids, and they were inoculated into groups of untreated normal eggs. This experiment gave a negative answer; treatment of the

infective inoculum did not result in the production of non-infective particles. The conclusion to be drawn from these two tests is clear: only periodate is effective in chemically inducing the von Magnus phenomenon, and its action is directed against the host tissue and not against the virus.

*Subsidiary Experiment II.*

Modification of receptors has been defined in the original description as any treatment of a receptive surface after which adsorption of influenza viruses continues unhindered while their spontaneous elution or artificial removal by receptor-destroying enzymes is prevented. To find out whether such modification was actually brought about by the standard periodate treatment, a separate experiment was set up to test the receptive properties of the allantoic wall.

Groups of six eggs were inoculated allantoically with 1·0 ml. of 0·01 M-$KIO_4$, or 0·01 M-$KIO_3$, or saline. Ten min. later each egg received 0·1 ml. 0·3 M glycerol, and after another 10 min. 0·60 ml. PR8 or LEE virus. Fifteen min. at 35° was allowed for adsorption, and a small sample (approx. 0·25 ml.) of fluid was then removed from the allantoic cavity; the haemagglutinin titre of this sample shows what proportion of the virus injected became attached to cells. To see whether the adsorbed virus could be eluted, 0·25 ml. of a receptor-destroying enzyme preparation (titre 100, by the method of Burnet and Stone) was injected and the eggs kept at 35° for a further 15 min. A second sample of fluid was then removed and titrated for haemagglutinin.

TABLE III.—*Adsorption and Elution of Virus after Various Pre-treatments of the Allantois.*

| Inoculum. | | Pre-treatment of allantois. | Average haemagglutinin titre after | |
|---|---|---|---|---|
| Strain. | Titre. | | Adsorption. | Elution. |
| PR8 (A) | 81 | $KIO_4$, 0·01mM. | 18·4 | 12·2* |
| | | $KIO_3$, 0·01mM. | 13·8 | 63·0 |
| | | Saline (control) | 16·1 | 74·3 |
| LEE (B) | 121 | $KIO_4$, 0·01mM. | 9·7 | 8·4* |
| | | $KIO_3$, 0·01mM. | 13·9 | 111·4 |
| | | Saline (control) | 14·5 | 102·3 |

* = Significant difference from respective control.

The results (Table III) show that none of the treatments interfered with the adsorption of the viruses to the allantoic wall, while $IO_4^-$ was the only reagent capable of preventing elution. It is concluded therefore that under the conditions of the main experiment cellular receptors have been modified by metaperiodate.

*Subsidiary Experiment III.*

Allantoic fluids containing non-infective particles often show lower infectivity than the seed from which they derived. This is particularly striking after artificial induction of the von Magnus phenomenon, as shown in the second half of Table I. Yet the objection that the harvested material was not the yield of a multiplication cycle, but rather had been left over from the original inoculum, could hardly arise. There are good, although indirect, arguments against this concept. The first is the increase in haemagglutinating particles, consistently present in all groups. Since this rise is between 10 and 100 times the original infective dose, and the cyclic increment of influenza viruses is of the same order, the simplest interpretation is to assume a multiplication cycle with a yield of about 99 incomplete and 1 complete virus

particle for each infective unit injected. The undiminished survival of the initial infective inoculum is rather unlikely also for another reason. Such doses of virus as used in our experiment are known to be readily ingested by cells (Fazekas de St.Groth, 1948) and indeed considerably larger ones undergo viropexis, as has been conclusively shown by Isaacs and Edney (1950) and by Cairns and Edney (1952). However, to furnish formal proof on this issue we have rinsed the allantoic cavity of eggs 3 hr. after infection, thereby removing all the virus free at that time. The allantois was then filled with gelatine-saline, and sampled a further 13 hr. later.

TABLE IV.—*Production of Incomplete Virus after Removal of Non-adsorbed Seed from the Allantois.*

| Inoculum. | | Treatment of allantois. | | |
|---|---|---|---|---|
| | | | After infection. | |
| Strain. | Dose ($ID_{50}$). | Before infection. | Nil. | Washing. |
| PR8 (A) | $10^9$ | Nil | 3·93* | 4·26* |
| | | $KIO_4$, 0·01mM | 3·12* | 3·20* |
| LEE (B) | $10^9$ | Nil | 6·29 | 6·21 |
| | | $KIO_4$, 0·01mM | 4·24* | 5·38* |

The figures show, in $\log_{10}$ units, the average ID/HA ratio of the yield.
* = Significant production of incomplete virus.

The results (Table IV) were as expected; the yield was undiminished and qualitatively indistinguishable from the control groups, *i.e.*, the same proportion of incomplete and infective particles was recovered as in the main experiment. It follows that the material harvested at the end of the usual incubation period is not the original seed but its offspring after at least one cycle of intracellular multiplication.

## CONCLUSIONS AND SUMMARY.

Theoretical considerations of the von Magnus phenomenon led to a new hypothesis on the mechanism of incomplete virus production. According to this the non-infective particles would lack an essential constituent normally provided by the host cell. This factor, tentatively identified with the receptor substance on the outside of the cell membrane, is believed to be transferred to the site of virus multiplication during viropexis.

The hypothesis was found to be consistent with existing data and in a crucial test it could be shown that allantoic cells were made to produce incomplete virus particles after chemical modification of their surface prior to infection. The agent employed, metaperiodate, acts on the cells and not on the virus. As predicted, after such treatment incomplete particles were yielded by the two strains representing the extremes of the incomplete virus gradient, and equally well after small and large inocula. Since incomplete virus particles could be produced hitherto only with some strains, and even with these only after concentrated inocula, chemical induction of the phenomenon provides a more general method of obtaining similar results.

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# SURVEY OF PAPERS.

HARRIS describes a method devised to give quantitative information about the multiplication of animal tissue cells *in vitro*. With it he has been able to measure the rate of growth of rat connective tissue cells in tissue culture, and to assess the effect of factors such as the size of the implant, oxygen tension and concentration of serum in the medium (p. 115).

DUTTON has inoculated mice with 10 species of pathogenic bacteria by three different routes and found that most of them were least lethal when injected intravenously, a finding apparently at variance with the commonly accepted idea that localisation helps to overcome infection. Two species of which the reverse was true were lethal only in very large doses, which are thought to have overwhelmed the reticulo-endothelial system when the intravenous route was used (p. 128).

ANDERSON has compared the iso-agglutinin response in rabbits immunised by repeated I.V. injections of saline suspensions of red cells with that obtained by antigen-adjuvant emulsion subcutaneously followed by a shorter I.V. course. A high iso-antibody titre against trypsin-treated cells was found in a greater proportion of animals submitted to the less time-consuming procedure (p. 137).

PARKER and CAMPBELL have found that when massive infarction of the liver is produced in rabbits by tying the hepatic artery, infection of the necrotic liver tissue by *Clostridia* does not follow. Such infection is said to result in dogs and to be preventable by giving penicillin, from which it has been argued that this antibiotic is indicated in similar clinical situations. The authors dispute the validity of this argument from one species to another and evidently believe that in this matter man behaves more like rabbit than like dog (p. 143).

MOLLIE BARR and MONA LLEWELLYN-JONES report further studies of mutual interference when two or more antigens are used for immunisation. These included experiments in which two single antigens were used, and others in which an injection of one was followed by a combination of this with another. The effect of the interval elapsing between different injections was particularly studied, and it seems that interference is unlikely to occur if this is one month or more (p. 147).

LODGE has shown that when rats are fed on glucose and saline only, without solid food, ulceration frequently occurs in the proventriculus and oesophagus. The gastric contents of these rats are very acid: that the cause of the ulceration is chemical injury was suggested by its much less frequent occurrence in rats also given bicarbonate. A circumferential excision of muscle to permit regurgitation into the oesophagus also caused ulceration there when acid and pepsin were fed. These lesions are believed to correspond to human oesophagitis occurring in conditions permitting reflux of gastric contents (p. 155).

BOYDEN and MARY E. ANDERSEN have found that when sheep red cells are added to mixtures of tuberculo-protein and the serum of an animal immunised with tubercle bacilli, agglutination results: if complement is added, haemolysis follows. The dilution of different immune sera producing this reaction varied, some being very weak: the globulins from such sera were more active than whole serum. Reasons are given for regarding this kind of haemagglutination as different from that occurring in the Middlebrook-Dubos test (p. 162).

HAVAS and JANET ANDRE have immunised mice with horse serum albumen in saline, or adsorbed to aluminium hydroxide or emulsified in Freund's adjuvant mixture. After four weekly I.P. injections, antibody measured by a quantitative precipitin method was highest in the Freund adjuvant group; an 8-week course showed aluminium hydroxide to be as

effective but, throughout, both these groups showed a high mortality. I.M. injections with Freund's adjuvant increased antibody production and also, at 4 weeks after an 8-week course, those with aluminium hydroxide (p. 171).

WIDDOWSON and MCCANCE have examined the enlarged hearts of anaemic piglets by mainly chemical methods, and adduce evidence that their enlargement is not due to hypertrophy in the usually accepted sense, but to an increase in the number of normal muscle cells (p. 175).

COOMBS, RUTH SAISON and VALERIE C. JOYSEY have studied, in different animal systems, co-agglutination resulting from the formation in a red cell suspension of a globulin-anti-globulin precipitate. The phenomenon was most marked with pig cells of which co-agglutination was produced with anti-pig globulin and a 1/100,000 dilution of pig serum. This spurious "agglutination" may complicate anti-globulin sensitisation tests when cells are inadequately washed (p. 179).

PAMELA M. WATERWORTH has studied the substances in blood which inactivate V factor and thus deprive it of capacity to support the growth of *H. influenzae*. There are two such substances, differing in distribution in the blood of different species, in thermolability and in activity when diluted: one occurs in the serum of horse blood but not in the cells, the second in the stroma of human cells but not in the serum. Sheep and ox blood contain both. The stroma substance is the more active, and a slight degree of haemolysis confers anti-V activity on the serum. A medium containing horse blood lysed with saponin is proposed as the most suitable for cultivating *H. influenzae* (p. 186).

PAGE THOMAS and DINGLE have determined the rate of oxygen uptake, glucose utilisation and lactic acid production *in vitro* of tissue removed from joints attacked by rheumatoid arthritis. Villi were more active metabolically than synovial membrane. Observations were also made on the degree of diminution of metabolic activity caused by the addition of different amounts of hydrocortisone (p. 195).

ROBERTS has found that when macrophages which have ingested typhoid bacilli are kept alive for several days, no agglutinin can be extracted from them. Macrophages were obtained from rabbits already immunised with typhoid bacilli, and the amount of agglutinin extractable from them was very small compared with that in the serum. It seems therefore that this cell can have little responsibility for antibody formation (p. 199).

FAZEKAS DE ST. GROTH and DORIS M. GRAHAM suggest that the production of incomplete influenza virus may depend on the inclusion within the cell, during viropexis in the first cycle, of an amount of cell membrane smaller than is required for the formation of infective virus. Experiments in support of this new concept include the demonstration that chick allantoic cells could be modified by metaperiodate so that even small inocula of a strain normally producing mainly infective virus from a large dose yielded incomplete virus (p. 205).

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